University of Alberta

Sulfur Amino Acid Metabolism in Parenterally and Enterally Fed Neonatal Piglets: The Effects of Gut Metabolism

by

Anna Katharine Shoveller

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in

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ABSTRACT

Sulfur Amino Acid Metabolism in Parenterally and Enterally Fed Neonatal Piglets: The Effects of Gut Metabolism

Anna Katharine Shoveller

University of Alberta, 2004

Co-Advisors

Ronald O. Ball and Paul B. Pencharz

The small intestine utilizes a portion of the dietary sulfur amino acids, methionine and cysteine; however, the effects of this utilization on parenteral SAA requirements and metabolism are not clear. Therefore, this thesis investigated the role of the neonatal small intestine in the metabolism of the sulfur amino acids.

The parenteral methionine requirement, when no cysteine was provided, was ~30% lower than the enteral requirement using the indicator amino acid oxidation technique. This suggests that the small intestine utilizes 30% of dietary sulfur amino acids. Using a similar design, but administering excess cysteine, we found that cysteine can replace 40% of the methionine requirement in both routes of feeding. Comparison of these studies demonstrated that cysteine is not an indispensable amino acid for protein synthesis in both enterally and parenterally fed neonatal piglets; however, plasma cysteine concentrations were lowest in the parenterally fed group receiving no cysteine. Furthermore, plasma homocysteine concentrations were higher in the enterally fed group receiving no cysteine than all other groups.

We then investigated N-acetyl-L-cysteine as a precursor of cysteine for neonatal piglets receiving total parenteral nutrition. N-acetyl-L-cysteine supported equal growth,

nitrogen balance and plasma urea as an isomolar amount of L-cysteine. Therefore, N-acetyl-L-cysteine is an effective precursor of cysteine in parenteral solutions for neonatal piglets.

The role of the small intestine in cysteine synthesis and oxidation was then investigated. Dietary cysteine reduced methionine oxidation, or cysteine synthesis through the transsulfuration pathway, until the total sulfur amino acid requirement was met, demonstrating cysteine sparing the methionine requirement. Comparison of the plateau in cysteine synthesis between enteral and parenteral routes of feeding demonstrated that parenterally fed piglets have a significantly greater rate of transsulfuration after the requirement for the total sulfur amino acids had been met. Therefore, the small intestine is not responsible for cysteine synthesis. Cysteine oxidation had a second order polynomial response to increasing dietary cysteine in enterally fed piglets, but had no association in parenterally fed piglets. There were no differences in cysteine oxidation between routes of feeding. Therefore, we believe that the gut exports homocysteine and plays an important role in the whole body metabolism of the sulfur amino acids.

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1.0 LITERATURE REVIEW

This review of the literature includes a description of sulfur amino acid metabolism focusing specifically on the metabolism of methionine and cysteine. The problems encountered in total parenteral nutrition (TPN) and neonatal nutrition, focused on the metabolism of the sulfur amino acids, will also be discussed. The piglet as a model for low birth weight infants in the study of nutrition will be discussed. This review will conclude with a discussion of previous studies which have estimated methionine and cysteine requirements for piglets and humans and methods to estimate the sulfur amino acid requirement and the capacity of cysteine to spare methionine.

1.1 Introduction

Premature birth is one of the leading causes of infant morbidity and mortality. One of the main goals in the care of these infants is providing them with an optimum nutritional profile in order to maximize growth, while attempting to keep metabolic stress low and sustain proper immune function. Premature and low birth weight infants often have complicating factors such as immature internal organs and short bowel syndrome, which make it difficult for them to receive enteral feeding (Ball et al, 1996). For the infant who cannot tolerate enteral feeding, TPN must be implemented. TPN involves the intravenous infusion of amino acids, glucose, lipids, vitamins and minerals and can be given alone or combined with enteral nutrition. Generally, the amino acid profiles of most TPN solutions are based on oral reference proteins and these may not be appropriate when first pass intestinal metabolism is by-passed or during periods of gut stress (Wykes et al, 1994, Ball et al, 1996, Brunton et al, 2000).

Although there have been great advances in TPN formulation, many problems remain. Generally, dietary protein is presented to the intestinal lumen as peptides and amino acids which are not simply absorbed into the systemic circulation but undergo metabolism within the enterocyte and liver (Heird, 1998). In order to minimize metabolic stress, identifying the optimum nutrient pattern for TPN is necessary. One of the challenges in defining parenteral amino acid requirements is to better understand to what extent some amino acids are synthesized or utilized within the enterocyte and in the liver during first pass metabolism, and what impact this has on amino acid requirements and/or metabolism. Therefore, the overall goal is to design a TPN solution based on research that has defined the indispensable amino acid requirements in parenterally fed neonates.

There is evidence that parenteral amino acid requirements are lower than enteral requirements due to the significant gut atrophy associated with parenteral feeding (House et al, 1998, Bertolo et al, 1998 and Elango et al, 2002). Stoll and colleagues (1998) demonstrated that the gastrointestinal tract (and specifically the small intestine), utilizes nearly 30% of amino acids that are supplied orally. Using portal mass balance, these researchers found that approximately 52% of enteral methionine was utilized by the gut and they could detect no cysteine in the portal blood. These results suggest that the gut has a specific requirement for the sulfur amino acids, but to what end remains unclear. In addition to the possible utilization of the sulfur amino acids by the splanchnic tissues, plasma total cysteine concentrations have been shown by numerous researchers to be lower during parenteral than enteral feeding (Miller et al, 1995, Van Goudoever et al, 1995, Stegink and DenBesten, 1972, Moss et al, 1999). Therefore, if gut atrophy causes

lower amino acid requirements, if methionine and cysteine are utilized by the splanchnic tissues, if cysteine utilization is higher during parenteral feeding or if cysteine synthesis is limited, then parenteral requirements for these amino acids may be significantly different from current oral recommendations.

Cholestatic jaundice and morbidity (Denno et al, 1996) are major complications for TPN fed neonates and the cause of this liver damage remains unknown. Moss et al (1993) demonstrated that an elemental solution itself, and not the route of alimentation, is hepatotoxic and this appeared to be mediated by one or more of the sulfur amino acids. In addition, methionine infusion, whether delivered enterally or parenterally, causes an increase in plasma homocysteine, a reduction in bile flow and bile excretion and histological evidence of liver damage (Moss et al, 1999). Increased plasma total homocysteine is associated with an increased risk of ischemic and hemorrhagic stroke in newborn infants and children (Hogeveen et al, 2002, Van Beynum et al, 1999). However, recent evidence suggests infusion of homocysteine, used as a cysteine precursor, increases bile flow but does not increase glutathione concentrations, synthesis or the level of oxidative stress (as defined by TBARS(thiobarbituric acid active substances)) in TPN fed rats (Belli et al, 2003). Therefore, it may not be the methionine, provided via parenteral administration, that causes cholestasis and other complications but the oxidative stress associated with reduced hepatic glutathione concentrations and parenteral administration of nutrients (Sokol et al, 1993) in neonates (Shahal et al, 1991).

In addition to the possible role that methionine plays in cholestasis, plasma cysteine concentrations are dramatically lower during parenteral compared to enteral feeding when cysteine or a cysteine precursor is not supplied. This suggests that either

cysteine synthesis is limited or cysteine utilization is higher in parenterally fed neonates. Complicating cysteine supplementation is the fact that cysteine is relatively unstable in solution and will form the insoluble dimer cystine if stored for a long period of time or subjected to heat sterilization (Heird, 1998). Although cysteine-hydrochloride was effective in increasing plasma cysteine concentrations (Zlotkin and Anderson, 1981), others have shown that supplementation of cysteine-hydrochloride to TPN results in metabolic acidosis (Laine et al, 1991). For these reasons, most commercial parenteral solutions contain very low (or no) cysteine (**Table 1.1**), and relatively high concentrations of methionine to meet the total sulfur amino acid requirement (Brunton et al, 2000), despite the virtual agreement that cysteine supplementation to TPN is necessary. Therefore, we must examine and understand how the neonatal small intestine and liver metabolize the sulfur amino acids, in order to improve TPN solutions and reduce the complications associated with parenteral feeding and sulfur amino acid toxicity or deficiency.

An optimum amino acid pattern for neonates receiving TPN needs to be empirically determined. However, clinical research involving premature infants is constrained by several factors: 1) a heterogeneous population with different complicating factors resulting in different metabolic demands, 2) ethical and practical constraints that limit sampling and different diet regimens and 3) current methods used in clinical situations may not be sensitive enough to detect small differences (Wykes et al, 1993). For this reason research has turned to using the piglet as an animal model for the human neonate. The neonatal piglet is considered to be an appropriate model for the human neonate because of many similarities in anatomy, physiology and metabolism

Table 1.1: Amino acid profile of commercially available neonatal amino acid solutions and human milk, sow's milk and human and pig bodies.

	Vamin (Pharmacia &Upjohn)[1]	Vaminolact (Pharmacia &Upjohn)[1]	Primene (Baxter)[1]	Aminosyn (Abbott)[1]	Travasol (Baxter)[1]	Trophamine (McGraw)[1]	Human milk[2]	Sow milk[3]	Human tissue[4]	Pig tissue[5]
Isoleucine	56	55	67	73	60	82	59	36	35	38
Leucine	75	108	99	95	73	140	108	88	75	72
Valine	61	55	76	81	58	78	61	50	47	52
Lysine	55	86	109	73	58	82	68	73	72	75
Methionine	27	20	24	40	40	34	16	16	20	20
Cysteine	20	15	19	0	0	1	NR[6]	NR	NR	NR
Phenylalanine	79	42	42	47	56	48	41	42	41	42
Tyrosine	7	8	9	9	4	23[a]	44	49	29	32
Threonine	43	55	37	52	4.2	42	49	48	41	37
Tryptophan	14	22	20	16	18	20	NR	NR	NR	NR
Histidine	35	32	38	30	48	48	25	33	26	28
Arginine	47	63	84	99	112	122	40	50	77	69
Glycine	30	32	40	129	103	36	27	34	118	91
Alanine	43	97	79	129	207	54	42	40	72	72
Aspartate	59	63	60	0	0	32	91	79	90	117
Glutamate	129	109	99	0	0	50	184	193	130	134
Proline	116	86	30	87	68	68	95	113	84	60
Serine	107	58	40	42	50	38	49	55	44	48
Taurine	0	0.5	6	0	0	2	--	--	--	--
Ornithine	0	0	22	0	0	0	--	--	--	--

[1]Values are **adapted** from Brunton et al, 2000. Values were originally presented as % of total amino acids by weight. Values have been converted to mg/g total amino acids for comparative purposes.

[2]Values are means of human milk determined in Davis et al (1993) and those calculated from Widdowson et al (1979), Renner (1983), Picone et al, (1989), Chavalittamrong et al (1981), Svanberg et al (1977), Janas and Picciano (1986), USDA and FAO (1972), and USDA (1976) and expressed as mg/g total amino acids. As presented in Davis et al (1993).

[3]Values reported by Elliot et al (1971) and expressed in mg/g total amino acids.

[4]Means of values from Widdowson et al (1979) in 160-180 d, 180-200 d, 200-220 d, 220-240 d, 240-260 d, and 260-280 d 6f age human fetus (mg/g total amino acids) as reported in Davis et al (1993).

[5]Means (mg/g total amino acids) of values from Williams et al (1954) in newborn, weanling, 33 kg and 93 kg pigs (g/16 g nitrogen), Wilson and Liebholz (1981) in 14, 28 and 35 d-old pigs (mg/g total amino acids) and Zhang et al (1986) in 3 and 9 week old pigs (g/16g nitrogen) as reported in David et al (1993).

[6]NR, not reported.

[a]Supplied as L-tyrosine (0.7%) and N-acetyl-tyrosine (1.6%).

(Benevenga, 1986, Miller and Ullrey 1987, Moughan and Rowan 1989).

The weaning time of piglets is becoming increasingly shorter in order to maximize piglets per sow per year and increase overall production. Currently, the National Research Council (1998) makes recommendations for piglets less than 5 kg based on extrapolations of studies on more mature pigs. When pigs are weaned early, the gastrointestinal tract does not have the digestive and absorptive capabilities to handle a normal post weaning grain based diet (Tokach et al, 1995). In addition, early weaning is often associated with gut stress. The combination of an immature gastrointestinal tract and gut stress results in poor nutrient utilization, post weaning growth lag and overall loss of money to the producer. To improve growth rates and overall dietary efficiencies, a more accurate nutrient profile needs to be established for piglets weaned at less than 5 kg. Therefore, we have chosen to use the neonatal piglet model to enable examination of sulfur amino acid requirements and metabolism in a model relevant to both the TPN fed human neonate and the early-weaned piglet.

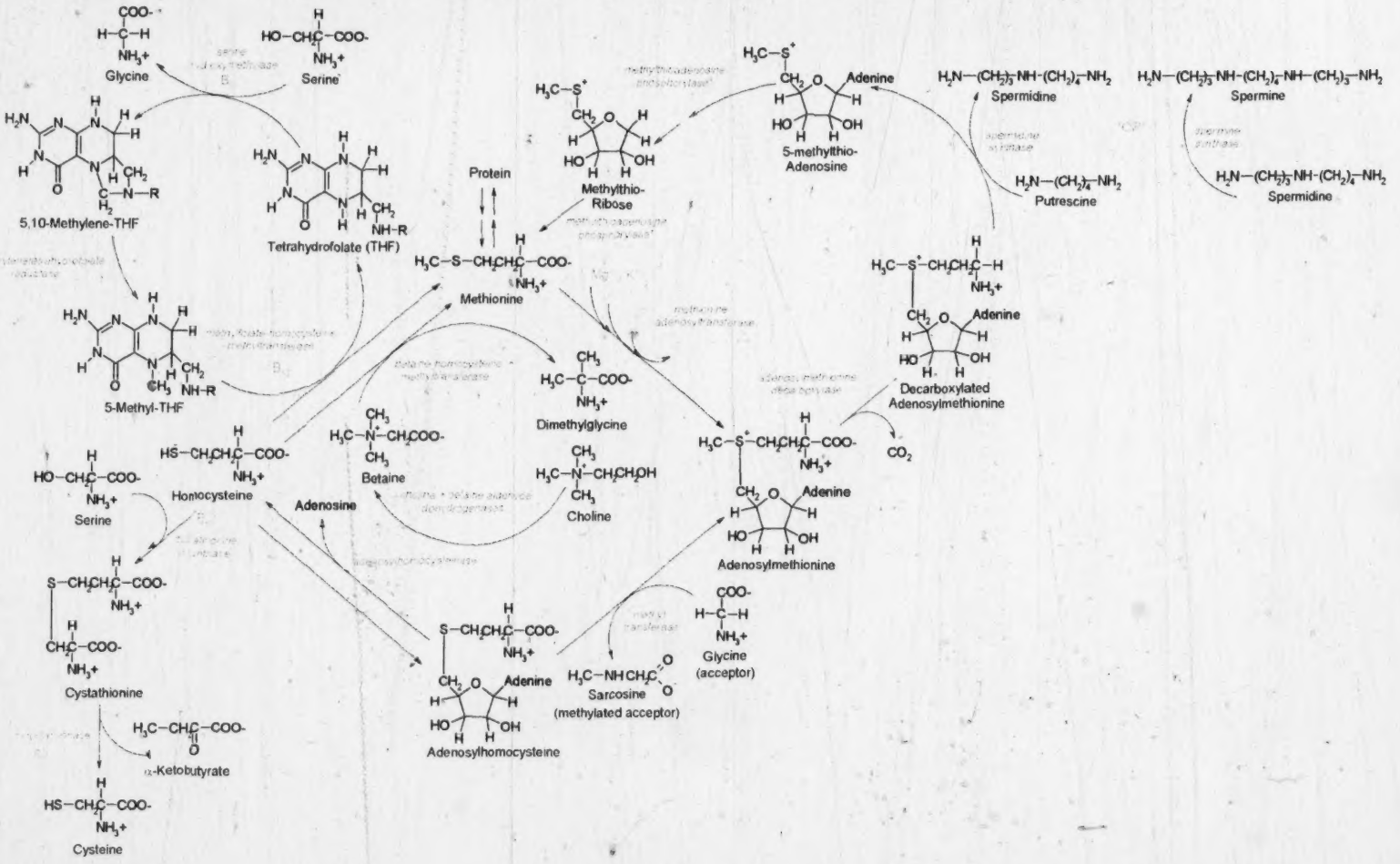
1.2 SULFUR AMINO ACID METABOLISM

1.2.1 Methionine metabolism in mammals

Methionine is an indispensable dietary amino acid and is needed for protein synthesis, methyl donation, and for the production of cysteine and further metabolites, taurine, sulfate and pyruvate. Methionine contains a thioether group (-S-CH_3) which is hydrophobic. Methionine can be metabolized to cysteine through the transsulfuration pathway, can be metabolized to CO_2 and SO_4 through the transamination pathway or can be converted through methylmalonyl CoA into succinyl CoA (Stryer, 1988).

The presence and significance of the transamination pathway has not been demonstrated in pigs or humans. However, the transamination pathway may be related to the marked toxicity of methionine in the rat (Steele et al, 1979) and deserves further investigation in other species. Therefore, the transsulfuration pathway is of greatest interest when examining the total sulfur amino acid requirement of pigs and humans and is represented along with transmethylation, remethylation pathways in **Figure 1.2.1.**. Methionine is converted to cysteine through a five-step process, which is regulated at the intermediate sulfur amino acid, homocysteine. In the first step of methionine metabolism, methionine and adenosine triphosphate (ATP) condense through the enzyme methionine adenosyltransferase (MAT; EC 2.5.1.6) to produce S-adenosylmethionine. S-adenosylmethionine is the primary methyl donor *in vivo*. There are at least three isoforms of mammalian MAT with the hepatic high K_m isoform (MAT III), hepatic

Figure 1.2.1: The transmethylation, remethylation, and transsulfuration pathways of methionine metabolism.



intermediate K_m isoform (MAT I) and the extraheptic low K_m isoform (MAT II) (for review see Finkelstein, 1990 and Stipanuk, 1986). Methionine partitioning between incorporation into protein and conversion to S-adenosylmethionine is the first regulatory site for methionine metabolism (Finkelstein, 1986).

In methyl transfer reactions, S-adenosylmethionine will transfer its methyl group to a variety of acceptors (Bender, 1985). The methyl donating capacity of methionine is important to the production of many compounds as well as an important mechanism to inactivate a number of active compounds such as catecholamines (phenylethanolamines, adrenaline, thymidine, lecithin and other compounds) (Bender, 1985). Although methionine is a net donor of methyl groups, glycine and serine are quantitatively the largest methyl donors. The major use of methionine methyl groups is the formation of creatine from guanidinoacetate (Mudd and Poole, 1975) and more recently, methyl group donation to the formation of phosphatidylcholine from phosphotidylethanolamine has been shown (Noga et al, 2003). Most methyl group losses and the removal of excess methyl groups can be accounted for by the production of sarcosine via the glycine methyltransferase (EC 2.1.1.20) reaction (Finkelstein, 1990).

S-adenosylmethionine is demethylated to produce S-adenosylhomocysteine via S-adenosylmethionine dependent transmethylation which utilizes numerous methyltransferases. S-adenosylhomocysteine is then hydrolyzed to produce adenosine and homocysteine through adenosylhomocysteine hydrolase (EC 3.3.1.1.). The formation of S-adenosylhomocysteine is thermodynamically preferred; however, the reaction is reversible and is inhibited by its products, homocysteine and adenosine (Finkelstein, 1990). Furthermore, hyperhomocysteinemia in rat results in a depletion of

plasma and tissue adenosine (Chen et al, 2002), although adenosine and homocysteine are produced simultaneously when S-adenosylhomocysteine is catabolized via adenosylhomocysteine hydrolase. Inhibition of this reaction results in the accumulation of adenosylhomocysteine and adenosylmethionine (Hoffman et al, 1980).

Homocysteine represents the second regulatory locus of methionine metabolism, as homocysteine may be remethylated to form methionine through either, betaine homocysteine methyltransferase (EC 2.1.1.5) or vitamin B-12 and folate dependent methylfolate homocysteine methyltransferase (EC 2.1.1.13) or may enter the irreversible transsulfuration pathway (Finkelstein, 1990). Remethylation of homocysteine to methionine is mainly dependent on the cofactors, folate, B-12 and betaine (Finkelstein, 2000) and on the demand for methionine. The regeneration of methionine from homocysteine or the conversion of methionine to cysteine largely depends on the state of the one-carbon pool and the relative tissue contents of both methionine and cysteine and the ratio of S-adenosylmethionine: S-adenosylhomocysteine (Finkelstein, 1990) and the choline supply. Therefore, the whole body concentration of methionine is inversely related to the rate of catabolism of one carbon units and the whole body concentration of methionine is directly associated with concentrations of S-adenosylmethionine.

For transsulfuration, homocysteine is irreversibly metabolized to cystathionine through pyridoxal 5'-phosphate, cystathionine β-synthase (CBS, EC 4.2.1.22). CBS is a heme protein (Kery et al, 1994) and has recently been shown to be up-regulated during oxidative stress, presumably to supply the cell with additional cysteine for glutathione synthesis (Mosharov et al, 2000). CBS activity is also regulated by the presence of dietary cysteine in an inverse relationship, either via sparing methionine catabolism via

protein synthesis, or by directly reducing CBS activity (Finkelstein, 1990). In the final step of the transsulfuration pathway, cystathionine is hydrolyzed to α-ketobutyrate and cysteine through pyridoxal 5'-phosphate-dependent γ-cystathionase (EC 4.4.1.1).

Methionine is also involved in polyamine metabolism. S-adenosylmethionine is irreversibly decarboxylated to decarboxylated adenosylmethionine via adenosylmethionine decarboxylase (EC 4.1.1.50). An amino propyl residue is donated from decarboxylated S-adenosylmethionine in the conversion of putrescine to spermidine, through spermidine synthase (EC 2.5.1.16). A similar reaction occurs when spermidine is converted to spermine, through the enzyme spermine synthase (EC 2.5.1.22). In these reactions, methionine is converted to methylthioadenosine, which is subsequently salvaged by conversion of methylthioribose moiety into methionine and the adenine moiety into adenine nucleotides (Backlund et al, 1981 and Schlenk et al, 1983). This is considered the methylthioadenosine salvage pathway (Stipanuk, 1986).

1.2.2 **Dietary influences on methionine metabolism**

Feeding cysteine resulted in a reduction in CBS activity, providing a mechanism by which dietary cysteine exerts its sparing effect upon methionine (Finkelstein and Mudd, 1967, Stipanuk and Benevenga, 1977). The reduction in CBS activity due to the addition of dietary cysteine can be due to cysteine sparing methionine for protein synthesis or cysteine synthesis. Others have used growth, feed efficiency and/or nitrogen balance to demonstrate that cysteine can replace part of the methionine requirement (Shelton et al, 1951, Curtin et al, 1952, Becker et al, 1955, Mitchell et al, 1969, Baker et al, 1969, Roth et al, 1989). More recently, a series of experiments examining the

response of methionine kinetics to varying intakes of methionine and cysteine suggested that there was no cysteine sparing mechanism in humans (Storch et al, 1990, Hiramatsu et al, 1994, Raguso et al, 1997, Fukagawa et al, 1998, Raguso et al, 1999 and Raguso et al, 2000). However, these studies provided the sulfur amino acids at a level consistent with the 1985 FAO/WHO/UNU recommendations, which DiBuono et al. (2001a and 2001b) have identified as being inadequate, at less than 50% of the total sulfur amino acid requirement. In order to demonstrate a response in methionine oxidation to addition of dietary cysteine, dietary methionine must first be able to meet its obligatory requirements.

The steps of transmethylation (methionine, SAM, S-adenosylhomocysteine and homocysteine) are present in all cells (Finkelstein, 2000). Alternatively, the transsulfuration pathway has limited distribution and is found primarily in the liver, kidney, small intestine and pancreas (Finkelstein, 2000). Transmethylation, remethylation and transsulfuration are all cytosolic reactions, while the oxidation of the carbon backbone of methionine (α-ketobutyrate) and cysteine (pyruvate) are oxidized via the mitochondrial tri-carboxylic acid cycle (Finkelstein, 1990). Although the formation of cysteine and its metabolites appears to be via a catabolic pathway for methionine, the transsulfuration pathway has the characteristics of a biosynthetic pathway, not a catabolic pathway. However, methionine supplementation to an otherwise adequate diet results in the reduction in the remethylation enzymes and an increase in the transsulfuration enzymes (Finkelstein and Mudd, 1967). The entrance of methionine into the transsulfuration pathway, although it can be reduced by cysteine, must be upregulated to handle high dietary intakes of methionine.

Levels of methionine 1.5 to 2 times the methionine requirement included in a low protein diet decreases feed intake, growth and nitrogen retention (Kade et al, 1948); levels above that will result in liver, kidney and pancreatic damage (for review: Harper, Benevenga and Wohlhueter, 1970). Other research has revealed that only the spleen is affected by high methionine intake, as evidenced by erythrocyte engorgement and an accumulation of hemosiderin (Benevenga et al, 1976). More recent data has shown that excess dietary methionine results in increased plasma total homocysteine and a decrease in bile flow and hepatobiliary function (Moss et al, 1999) and these precede cholestasis during parenteral feeding. Methionine is more toxic than homocysteine and cysteine; therefore, it is likely that methionine toxicity is due to the additive effects of excess methionine accumulation, methyl group accumulation, homocysteine accumulation and cysteine accumulation (Harper, Benevenga and Wohlhueter, 1970). Furthermore, these toxic effects are likely compounded by the depletion of the whole body glycine and serine pools, as supplements of these amino acids significantly enhance the rate of oxidation of methionine in rats adapted to a high methionine (Benevenga and Harper, 1970) or homocystine (Benevenga and Harper, 1967) diet. This suggests that the supplementation of these amino acids allowed for upregulation of methionine oxidation by increasing the flux through the transsulfuration pathway to avoid buildup of both methionine and methyl groups. In contrast to a control diet, a high methionine diet caused a marked increase in sulfur excretion and cysteine dioxygenase, cysteine desulfhydrase, cystathionase and cysteine aminotransferase activity, demonstrating that there is increased flow of methionine sulfur through the cysteine degradative pathways (Stipanuk, 1979). What remains unclear is the fate of excess dietary methionine when

cysteine is supplemented in the diet. Supplementation of dietary cysteine (DiBuono et al, 2003) and glutathione (Fukagawa et al, 1996) has been shown to decrease transmethylation and transsulfuration and increase remethylation in adult humans. Indeed, early work by Finkelstein and Mudd (1967) demonstrated that there was a 35% depression in the activity of MAT and a 45% depression in the activity of cystathionine β-synthase in liver extracts from rats fed a low methionine diet supplemented with cystine, compared with extracts from rats fed a low methionine diet without dietary cysteine. Further research is required to understand the pathophysiology of sulfur amino acid toxicity and the fate of methionine when cysteine is supplied in the diet.

1.2.3 **Cysteine metabolism in mammals**

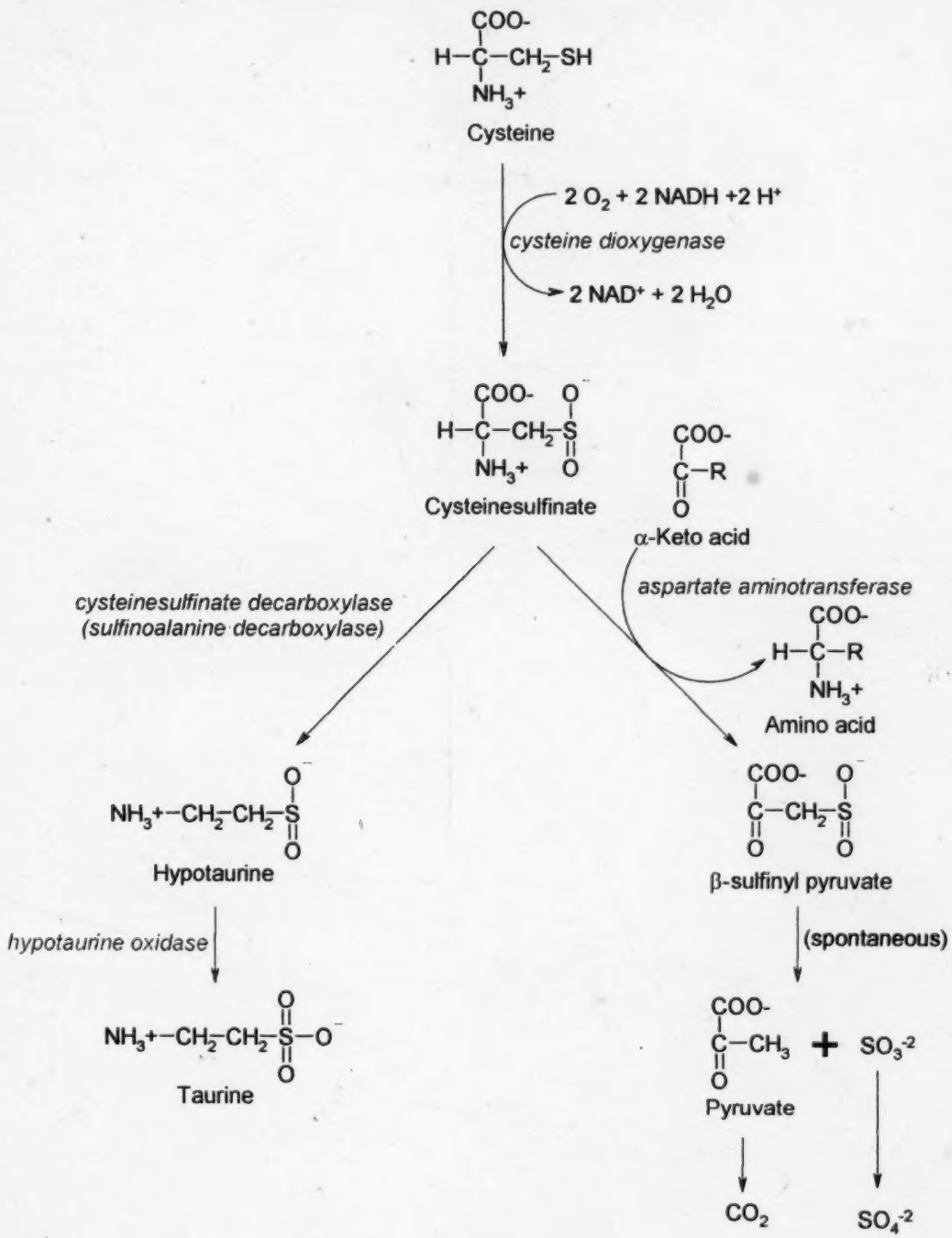
Cysteine and the dimer of cysteine, cystine, are conditionally indispensable amino acids for mammals, because cysteine can be synthesized from the sulfur moiety of methionine and the carbon backbone of serine through the transsulfuration pathway if adequate methionine is provided in the diet. Cysteine contains a sulfhydryl group (-SH) which is hydrophobic and highly reactive. Cysteine is needed for the production of taurine, sulfate, and glutathione. Cysteine is constantly undergoing oxidation/reduction reactions to form disulfide bonds with albumin, homocysteine and cysteine, due to this highly reactive sulfhydryl group. As such, cysteine is present in the plasma almost completely in the oxidized form (cystine) and in the reduced form intracellularly due to the intracellular reducing environment (Crawhall et al, 1967).

Cysteine is catabolized *in vivo* to yield either taurine and CO_2, or sulfate, urea and CO_2, whether synthesized from methionine and serine, or exogenously supplied in the

diet (Krijgsheld et al, 1981). The cysteine sulfinate pathway of cysteine metabolism is depicted in **Figure 1.2.3.1**. Through the addition of oxygen and the action of cysteine dioxygenase (EC 1.13.11.20), cysteine produces cysteine sulfinate which is completely oxidized. Approximately 70-90% of cysteinesulphinate is decarboxylated via cysteine sulfinate decarboxylase (EC 4.1.1.29) to produce hypotaurine, which is then oxidized to taurine via a poorly characterized enzymatic reaction (Yamaguchi et al, 1973, Griffith, 1982, Stipanuk and Rotter, 1984). The cysteine sulfinate pathway is the major synthetic pathway for taurine in mammals; however, cysteine sulfinate decarboxylase activity is limited in the cat (Hayes et al, 1975), monkey (Hayes et al, 1980) and man (Jacobsen et al, 1968). Cats (low cysteine sulfinate decarboxylase activity) and rats (high cysteine sulfinate activity) oxidize similar amounts of cysteine, but cats oxidize more cysteine sulfinate to pyruvate (80%) than the rat (De La Rosa and Stipanuk, 1985). These data are consistent with the enzyme data for cysteine sulfinate decarboxylase among mammalian species. Alternatively, cysteine sulfinate may also be catabolized to taurine through cysteic acid, but this is believed to be a relatively minor biochemical pathway (Stipanuk, 1986).

Alternatively, cysteine sulfinate may undergo transamination or oxidative deamination to form the putative intermediate, β-sulfinylpyruvate, which spontaneously decomposes to yield pyruvate and sulfite (Singer et al, 1956); this pathway accounts for 10 to 30% of cysteinesulfinate degradation.

Figure 1.2.3.1.: The cysteinesulfinate dependent catabolism of cysteine



1.2.4 **Taurine metabolism in mammals**

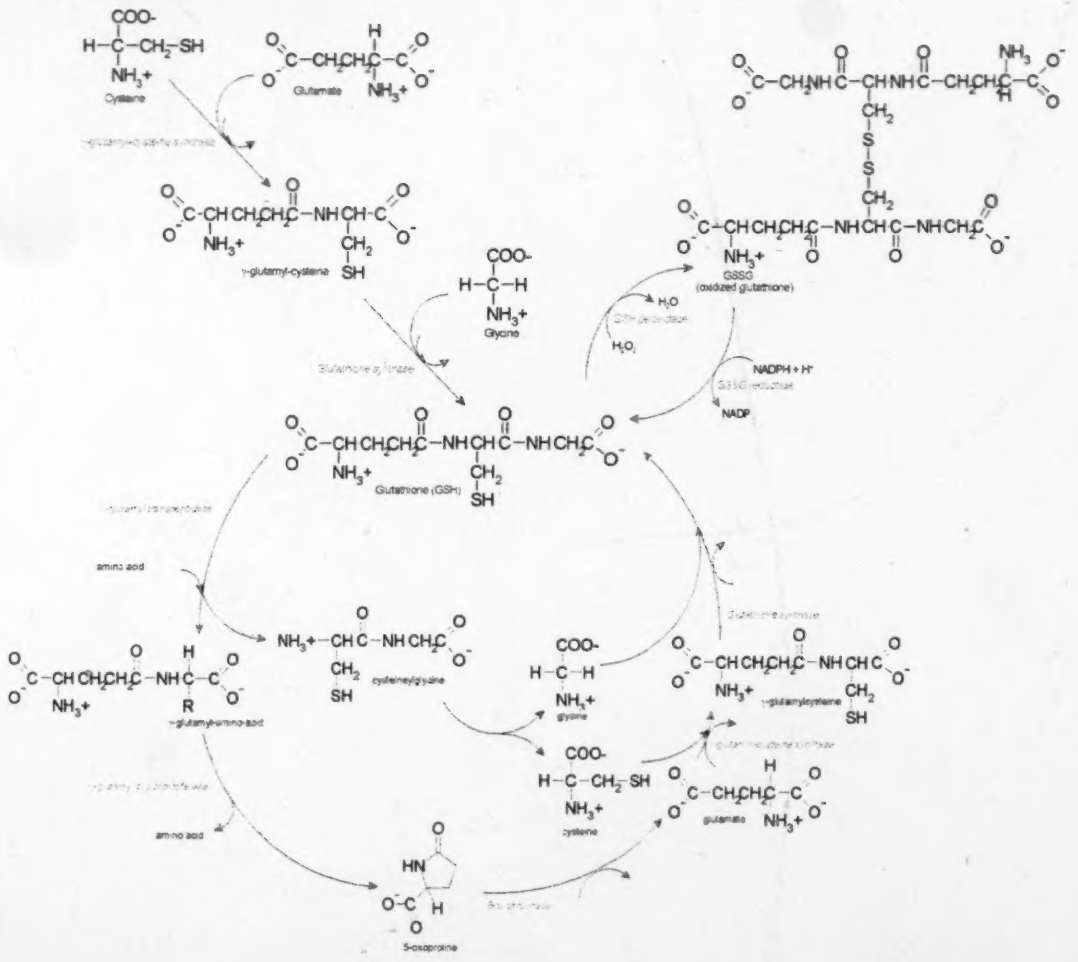
Taurine (2-aminoethanesulfonic acid) is a beta amino acid, not incorporated into protein. Taurine is synthesized from cysteine through the cysteine sulfinate pathway (Stipanuk, 1986), as described above. The major fate of taurine is conjugation with cholic acid to produce taurocholic acid which is excreted with bile, aids in the digestion of lipids in the intestinal lumen (Hayes and Sturman, 1981) and is ordinarily found in tissues in its free form. Taurine is relatively absent in plants and therefore is mainly derived from animal products in the diet (Hayes and Sturman, 1981).

1.2.5 **Glutathione synthesis and metabolism**

Gluthathione is a tripeptide of glycine, glutamate and cysteine and contains a sulfhydryl group that is mainly thought to protect red cells from oxidative damage. The intracellular formation of glutathione (**Figure 1.2.5.1**), requires two enzymes, γ-glutamyl cysteine synthase and glutathione synthase which both also require adequate ATP (Meister and Anderson, 1983). A tripeptide linkage is formed between the γ-carboxyl group of glutamate and the amino group of cysteine and catalyzed by γ-glutamyl cysteine synthase. The reaction is inhibited by glutathione in a negative feedback mechanism. Glutathione synthase then catalyzes the second and last reaction in which ATP activates the carboxyl group of cysteine to enable it to condense with the amino group of glycine (Meister and Anderson, 1983).

Glutathione has been found at high levels in animal cells, and is believed to serve in a variety of functions, such as synthesis of proteins and DNA, transport of cysteine,

Figure 1.2.5.1: Glutathione synthesis and metabolism



amino acid transport, enzyme activity, as an antioxidant and in drug metabolism (Meister and Anderson, 1983). As such, glutathione fluctuates between the reduced (GSH) and the oxidized form (GSSG) both extra and intracellularly (Stryer, 1988). Under normal conditions, the majority of glutathione is found in the reduced form; however, under conditions of marked toxicity or oxidative stress, intracellular GSSG increases substantially (Meister and Anderson, 1983).

Glutathione and the glutathione synthetic enzymes are found in portions of the small intestine and in the gastric mucosa. Glutathione is important as a first line of defense in the gut. Intestinal changes in glutathione content have been related to an increased susceptibility to carcinogenesis, oxidative injury, metal intoxication and common intestinal pathologies (Iantomasi et al, 1997).

1.2.6 **Regulation of cysteine metabolism**

The whole body oxidation of cysteine in rats accounts for approximately 40% of the whole body cysteine flux (Stipanuk and Rotter, 1984), suggesting that the remaining 60% contributes to protein and glutathione synthesis. Rats fed diets containing excess cysteine compared to rats receiving methionine only, have higher hepatic cysteine dioxygenase activity and lower cysteinesulfinate decarboxylase (Stipanuk and Rotter, 1984) and γ-glutamylcysteine synthetase activity (Ohta et al, 2000). Furthermore, cysteine dioxygenase activity increased and γ-glutamylcysteine synthetase activity decreased in a dose-response manner in cells cultured in either methionine or cysteine supplemented media, while cysteinesulphinate decarboxylase activity did not change with sulfur amino acid supplementation (Ohta et al, 2000, Kwon and Stipanuk, 2001).

Furthermore, supplementation of the culture media with cysteine yielded responses of greater magnitude than did supplementation with an isomolar amount of methionine (Kwon and Stipanuk, 2001). Bagley and Stipanuk (1994) demonstrated that rats fed excess protein as casein, had higher CDO activities than rats fed a control diet, likely in response to increased amino acid degradation. The increased activity of CDO seen in high protein feeding may also be related to the different hormonal and metabolic state of these animals (Bella and Stipanuk, 1996). Although CDO activity is increased and γ-glutamylcysteine synthetase activity decreased with increases in cysteine, these enzymatic changes do not affect glutathione concentrations (Kwon and Stipanuk, 2001), but do decrease glutathione efflux from incubated hepatocytes (Bagley and Stipanuk, 1995). Moreover, although there is a pronounced increase in CDO activity when dietary cysteine is increased, Stipanuk and Rotter (1984) showed in rats that diets with adequate or excess cysteine did not affect the appearance of $^{13}CO_2$ from C1 (released during taurine and pyruvate oxidation) and C3 (released during pyruvate oxidation) labeled cysteine. Few in vivo experiments have been done since to explain the apparent differences in the vitro enzymatic data and the in vivo oxidation data. To our knowledge, there have been no studies examining cysteine catabolism in parenterally fed animals. Given the speculation that cysteine is conditionally essential during parenteral feeding, the fate of dietary cysteine needs to be examined.

1.2.7 **Sulfur amino acid metabolism in the neonate and during parenteral feeding**

When TPN is administered for a prolonged period of time, gut atrophy is induced (Adeola et al, 1995, Goldstein et al, 1985) and protein synthesis is reduced (Dudley et al,

1998). Although the portal drained viscera comprises 3-6% of total body weight, it is responsible for 15% of whole body protein synthesis (McNurlan and Garlick, 1980) and 20 to 35% of energy expenditure (Stoll et al, 1997). Therefore, the maintenance of the gastrointestinal tissue is mainly dependent upon enteral feeding.

As mentioned in the introduction, administration of TPN by-passes first pass intestinal and hepatic metabolism. During intravenous and intraportal infusion of nutrients, piglets have lower nitrogen retention and intestinal mucosal weights as compared to gastric feeding; however, only intravenously fed piglets had lower crypt depths when compared to gastrically fed piglets (Bertolo et al, 1999). This suggests that small intestinal atrophy affects nitrogen metabolism to a greater extent than liver by-pass. Furthermore, intravenous and intraportal feeding resulted in reduced concentrations of numerous amino acids in plasma and tissues (Bertolo et al, 2000), further highlighting the importance of intact first pass intestinal and hepatic metabolism on amino acid metabolism. There is also evidence that by-passing first pass splanchnic metabolism may impact amino acids requirements. Using the indicator amino acid oxidation technique, both the threonine (Bertolo et al, 1998) and branched chain amino acid requirement (Elango et al, 2002) have been shown to be significantly lower during parenteral than enteral feeding. This is supported by Stoll et al (1998) who measured the appearance of labeled amino acids in the portal vein of piglets following an intragastric infusion of [U-^{13}C] algal protein. These researchers found that approximately one third of the dietary indispensable amino acids were consumed in first pass metabolism by the intestine and this difference cannot solely be due to protein synthesis (Stoll et al, 1998). Furthermore, only 48% of the infused methionine, and no cysteine, was found in the

portal blood, suggesting a net utilization of 52% of the infused methionine (Stoll et al, 1998). Ebner et al (1994) also found a net uptake of methionine by the portal drained viscera. These data suggest that the sulfur amino acids are selectively utilized by the splanchnic tissues and if so, would result in the parenteral requirement for the sulfur amino acids being lower than the enteral estimates. It remains to be elucidated whether the apparent utilization of the sulfur amino acids results in a difference between the enteral and parenteral total sulfur amino acid requirement.

Whether or not cysteine is an indispensable amino acid in parenterally fed neonates remains controversial. In neonatal animals, the slow maturation of the enzyme cystathionase may limit *de novo* cysteine synthesis (Pascal et al, 1972, Sturman et al, 1970, Gaull et al, 1972). However, cystathionase activity is present in both the adrenals and kidneys of both premature and term infants, suggesting that term infants may not require additional cysteine. The most convincing evidence that cysteine may be indispensable during parenteral feeding comes from the fact that in neonates (Van Goudoever et al, 1995, Zlotkin et al, 1981) and rabbits (Moss et al, 1999), feeding parenterally causes a dramatic reduction in plasma cysteine. However, Zlotkin et al (1981) demonstrated that infants (n=28, n=17 premature infants and n=11 full term infants) receiving supplemental cysteine to a parenteral solution (n=18) had higher plasma cysteine concentrations than infants not receiving supplemental cysteine (n=18) but the supplemental cysteine did not improve nitrogen retention. Alternatively, if supplemental cysteine does not improve nitrogen retention and does not improve protein synthesis, then cysteine may be metabolized to other products such as taurine, glutathione or pyruvate. After a cysteine infusion, serum cystine concentrations decrease

to pre-infusion concentrations within 6 hours, while sulfate concentrations remain elevated for 24 hours (Krijgsheld et al, 1981). It is possible that cysteine is temporarily stored in a labile glutathione pool. Indeed, beagle pups that received cysteine supplemented TPN had similar growth rates but significantly greater plasma cysteine and hepatic glutathione and cysteine concentrations than beagle pups that received TPN with methionine only (Malloy and Rassin, 1984). Compared to the chow fed controls, parenterally fed beagle pups receiving supplemental cysteine had similar total plasma cysteine and hepatic glutathione concentrations (Malloy and Rassin, 1984). Furthermore, plasma taurine concentrations did not differ between parenterally fed beagle pups that were or were not supplemented with cysteine but both parenterally fed groups had lower plasma taurine than the chow fed controls (Malloy and Rassin, 1984). Therefore, even in animals that have sufficient enzymatic activities to synthesize cysteine from methionine, an exogenous source of cysteine may be necessary during parenteral feeding to support glutathione and taurine synthesis. Clearly, the question of dietary indispensability of cysteine and the reason for the low plasma concentrations of cysteine requires further investigation.

Because of the low plasma cysteine concentrations that are observed during parenteral feeding, methionine, may be over-supplemented and thus, may be toxic. Moss et al (1993) demonstrated that parenteral feeding in the rabbit is directly hepatotoxic and this toxicity appeared to be mediated by one or more of the sulfur amino acids. Further investigation demonstrated that both total parenteral nutrition and enteral feeding with an intravenous methionine infusion resulted in decreased hepatobiliary function and bile flow and increased plasma total homocysteine (Moss et al, 1999). Further investigation

of the role of sulfur amino acids in TPN induced cholestasis is required; however, first the requirements and metabolism of the sulfur amino acids during parenteral feeding need to be defined. Providing methionine and cysteine at the requirement and in the optimum ratios may reduce this problem.

Although taurine is not required for body protein synthesis, prolonged deficiency may lead to retinal abnormalities in children (Geggel et al, 1985). Taurine synthesis may be impaired in neonates due to the low or absent cysteine sulfinate decarboxylase activity and increased renal taurine losses (Helms et al, 1995) in humans, monkeys and cats (Hayes and Sturman, 1981). The rat has high levels of cysteinesulphinate decarboxylase activity compared to the cat or human (De La Rosa and Stipanuk, 1985) and as such, the rat mainly conjugates bile acids with taurine, not glycine. Such a correlation between cysteine sulfinate decarboxylase and bile conjugation, does not apply to cats and primates, who have limited cysteine sulfinate decarboxylase activity and conjugate bile acids mainly with taurine. This is one of the reasons why taurine is a dietary indispensable amino acid for primates and cats. In low birth weight infants, conjugation of bile acids with taurine exceeds that with glycine for the first three weeks of life, but this may be largely due to the low ratio of glycine: taurine ratio in human milk (Brueton et al, 1978). Therefore, taurine may be required in neonates due to the low cysteinesulfinate decarboxylase activity in this population. Furthermore, if cysteine synthesis is impaired in neonates, cysteine and taurine supplementation would both need to be considered.

Finally, glycine may be the first limiting amino acid for growth in infants on low protein diets, as weight gain is increased by dietary supplementation (Snyderman et al,

1962). During growth the total requirement (g/d) and the relative requirement (% of total amino acids) for glycine is high (Jackson, 1991). The neonate will conserve glycine by reducing its degradation (Jackson et al, 1981) and limiting its use for bile acid synthesis. Therefore, glycine status can be compromised if either the ability to form glycine from its precursor serine is constrained or the demand for glycine is increased. If glycine is in fact a conditionally indispensable amino acid for low birth weight infants, a deficiency in glycine could very well perturb sulfur amino acid metabolism. Glycine deficiency could lead to increased susceptibility of methionine toxicity due to a perturbation in the methyl-folate trap, reduced glutathione synthesis and perturbed bile acid conjugation if taurine can not meet this need alone. Clearly, glycine could affect many facets of sulfur amino acid metabolism.

1.3 N-acetylcysteine as a precursor of cysteine in parenteral solutions

Despite the well accepted belief that cysteine is conditionally indispensable in parenterally fed neonates, few commercial parenteral solutions contain appreciable amounts of cysteine and others contain no cysteine at all (Table 1.1). This is mainly due to the instability of cysteine in all heat sterilized solutions (Heird, 1998). Given the toxic effects of methionine and parenteral feeding, the low plasma cysteine concentrations that are found during parenteral feeding and the relative instability of cysteine in solution, alternatives to cysteine supplementation in parenteral solutions are necessary. Various forms of cysteine have been suggested for inclusion in parenteral solutions. Cysteine hydrochloride may be added to parenteral solutions (Zlotkin et al, 1981), but has resulted

in metabolic acidosis (Laine et al, 1991). Another approach is to supplement a stable precursor of cysteine that can be converted to cysteine *in vivo*.

N-acetyl-L-cysteine is soluble and stable in solution and it is also resistant to Maillard destruction (Baker et al, 1984), thus enabling heat treatment of the solution. This approach is based on the rationale that N-acetyl-L-cysteine will be deacylated in vivo to cysteine. N-acetyl-L-cysteine is deacetylated via the enzyme aminoacylase I (EC 3.5.1.14). Aminoacylase I is a cytosolic enzyme (Sjodin et al, 1989) present in the liver, kidney, endothelial tissue, lung and intestinal mucosa and its highest activity occurs in the kidney in the rat (Yamauchi et al, 2002 and Endo, 1980), pig (Henseling et al, 1988), and human (Yamauchi et al, 2002). In the pig, N-acetylmethionine and N-acetyl-L-cysteine are natural substrates for acylase I (Giardina et al, 1997). When N-acetyl-L-cysteine was added to rat liver, lung and intestine homogenates, nearly stoichiometric amounts of L-cysteine were recovered (Sjodin et al, 1989) demonstrating that N-acetyl-L-cysteine is readily deacetylated to cysteine. Given that acylase I is efficient at *in vitro* deacetylation of N-acetyl-L-cysteine, the deacylation of N-acetyl-L-cysteine to L-cysteine should result in metabolically available L-cysteine *in vivo*.

Neuhauser et al (1986) demonstrated that rats receiving total parenteral nutrition that supplied methionine as the sole source of the sulfur amino acids had similar nitrogen balance and growth as rats that had partial replacement of methionine by N-acetyl-L-cysteine. In addition, only 4.6% of the total infused N-acetyl-L-cysteine was detected in the urine of these rats, suggesting that N-acetyl-L-cysteine was approximately 95% available. Baker and Han (1993) found that young rats fed a casein based oral diet supplemented with either L-cysteine or N-acetyl-L-cysteine achieved similar growth

rates. In adult humans receiving a 4 hour intravenous infusion N-acetyl-L-cysteine, 11% of the infused amount was detected in the urine (Magnusson et al, 1989), suggesting that N-acetyl-L-cysteine is 89% bioavailable in the adult human.

Although growing and adult rats and adult humans seem to be efficient at the deacylation of N-acetyl-L-cysteine, data in neonates is sparse. VanGoudoever et al (1994) administered three different amino acid solutions, one (Aminovenos, Fresenius AG) of which contained a low (5 mg/ g protein) concentration of N-acetyl-L-cysteine, while the other solutions contained free base L-cysteine, to premature neonates (n= 10 in the treatment receiving Aminovenos (Fresenius, AG) with N-acetyl-L-cysteine, n=4 in the treatment receiving Vaminolact (Kabi Pharmacia) and n=6 in the treatment receiving Primene (Clintec)) for 5 days. These researchers found more than 50% of the infused N-acetyl-L-cysteine was excreted in the urine while plasma cystine concentrations remained low. They concluded that N-acetyl-L-cysteine was a poor precursor to cysteine in parenterally fed neonates, even though the N-acetyl-L-cysteine group had similar rates of growth and nitrogen retention compared to the other solutions which contained cysteine. In addition, these researchers did not present urinary excretion rates of N-acetyl-L-cysteine and L-cyst(e)ine and the infants received low infusion rates compared to other studies in adult humans. The efficiency of conversion of N-acetyl-L-cysteine to cysteine in the neonate needs to be established, as it is unclear whether the parenterally fed neonate has the ability to utilize this precursor.

1.4 The piglet as a model for the premature infant

Premature birth is a leading cause of infant morbidity and mortality. Appropriate nutritional strategies need to be developed to care for these infants who are often unable to tolerate oral feedings due to the many complications associated with premature birth. Total parenteral and partial parenteral nutrition are often necessary for these infants. In order to promote healthy growth, as well as avoiding an excessive supply of nutrients, which will put undue stress on immature organs, optimal nutrition for premature infants needs to be determined.

The empirical determination of nutrient requirements and nutrient metabolism in this population is constrained by many ethical and practical constraints. The premature or low birth weight infant population is extremely susceptible to complications, including infection, which can dramatically change their metabolic demands. The limitations of sampling of blood, breath and urine also impose constraints upon the researcher and limit the area of study dramatically (Ball et al, 1996). Application of an animal model enables the study of a homogenous population, thus allowing the use of more invasive and sensitive methods. Finally, a subset of premature infants would be highly diverse and make it difficult to elucidate the effects of small, but important changes in the metabolic handling of dietary nutrients (Ball et all, 1996).

Most of the research examining sulfur amino acid metabolism has been done in the rat; however, rat metabolism is very different from human metabolism and is difficult to examine in neonatal rats because they are born very immature and small. Unlike the rat, swine have many physiological, anatomical and biochemical similarities to humans which makes them a very good model for the human neonate (Benevenga, 1986). The

current estimated amino acid requirements for piglets (NRC, 1998) are similar to those recommended for the human neonate, when represented as a % of total amino acids (Ball et al, 1996). However, the piglet grows at a faster rate than the human neonate; therefore, experiments examining amino acid metabolism are sensitive and can be accomplished in a short amount of time. Results from these studies are also beneficial to the swine industry and can be directly applied to better understanding the nutrient metabolism of the early weaned piglet.

1.5 Sulfur amino acid requirements in neonates

Experimentally derived estimates of total sulfur amino acid requirements or the bioavailability of precursors of these amino acids have been obtained by several methods. These include growth, nitrogen balance, and indicator amino acid oxidation. All of these methods are based on the fundamental principle that if one indispensable amino acid is less than adequate, then protein synthesis is limited by the limiting amino acid. Using the direct oxidation technique to measure the methionine requirement is difficult since infusing 1-[^{14}C]-methionine will not measure protein synthesis because the oxidation of the 1-carbon is only possible after the entrance of methionine into the transsulfuration pathway and the subsequent release as α-ketobutyrate upon the synthesis of cysteine. Therefore, using the direct amino acid oxidation technique will only give an estimate of the flux of methionine through the transsulfuration pathway. The indicator amino acid oxidation technique is the only isotopic method that would provide a good estimate of the total sulfur amino acid requirement.

To our knowledge, there are no published studies in which the total sulfur amino acid requirement and the ability of cysteine to spare the methionine requirement have been directly measured in human neonates. Previous estimates of the sulfur amino acid requirements are shown in **Table 1.5.1**. There are however, studies that have determined the total sulfur amino acid requirement in growing pigs (<5 kg body weight) by measuring growth, nitrogen balance, plasma free amino acids, or plasma urea nitrogen (Table below) and these, when examined with similar diets, are surprisingly similar. This indicates that the total sulfur amino acid requirement is likely between 0.40-0.70 % of the diet. Furthermore, in growing pigs, the maximal proportion of sulfur amino acid requirement that can be met by cysteine has been estimated to range between 40 and 70% (Shelton et al, 1951, Curtin et al, 1952, Becker et al, 1955, Mitchell et al, 1968, Baker et al, 1969, Roth and Kirchgessner, 1989 and Kim and Bayley, 1983). Kim and Bayley (1983) measured the total sulfur amino acid requirement in 10 to 14 day old piglets (~ 3kg) and estimated the total sulfur amino acid requirement to be 0.43 g kg^{-1} d^{-1} and the methionine requirement when excess cysteine is present to be 0.25 g kg^{-1} d^{-1}. Chung and Baker (1992) demonstrated that 10 kg pigs receiving a 60:40 and 50:50 ratio of methionine: cystine had higher growth rates than those fed 40:60.

Snyderman et al (1964) estimated the requirement of methionine, with 2.14% cysteine/ total amino acids, in 4 term and 3 premature infants. These researchers concluded that the requirement for methionine was between 0.85-1.34% methionine/total amino acids, totaling 3.48% total sulfur amino acids/total amino acids. However, half the infants in the study had met their maximum nitrogen balance at the lower end, suggesting the requirement was lower than estimated. Fomon et al (1986) measured the

Table 1.5.1: Estimates of sulfur amino acid requirements in neonatal piglets and humans infants.

Estimated TSAA requirement	species	Methionine: Cyst(e)ine Ratio	weight	Type of diet	Dependent variables	references
0.50-0.70(% of diet)	Pig		5	Semi-purified	Growth, feed efficiency, nitrogen balance	Kroening et al, 1995
0.44 (% of diet)	Pig	70:30	1-5	Semi-purified	Oxidation of indicator amino acid	Kim and Bayley, 1983
0.60 (% of diet)	Pig		2	Semi-purified	Growth, feed efficiency, nitrogen balance	Leibholz, 1984
3.48% of total amino acids	Human neonate	40:60		Enteral formula	Nitrogen balance and weight gain	Snyderman et al, 1964
2.37% of total amino acids	Human neonates	45:55		Isolated soy protein formula	Nitrogen balance, plasma urea, plasma albumin, growth	Fomon et al, 1986

sulfur amino acid requirement in infants 6 to 112 days of age and found that no more than 2.37% of total amino acids were needed when the methionine intake was 1.07% of total amino acids. Clearly, there is a discrepancy in the estimate of the requirement for the total sulfur amino acids and the ability for cysteine to spare the methionine requirement. To clearly define these requirements, a properly designed requirement study is necessary. To our knowledge, no one has directly measured the total sulfur amino acid requirement or the methionine sparing capacity of cysteine in parenterally fed infants.

1.6 Design, analysis and interpretation of requirement experiments

To properly assess amino acid requirements, one must give careful attention to the experimental design, statistical analysis implemented, and interpretation of the results. Most experiments that have defined the sulfur amino acid requirements have used growth, feed efficiency or nitrogen balance. To allow sufficient statistical power, both requirement and bioavailability studies necessitate feeding graded levels of the amino acid in question (Baker, 1986). A minimum of three dietary levels of the amino acid in question (independent variable), and preferably six or more, is required for the data to be fitted to a descriptive response, which allows objective assessment of the requirement (Baker, 1986). It is important to note that the statistical differences, or lack there of, between any two adjacent diet levels are essentially meaningless (Baker, 1986). Therefore, applying a continuous broken line model (the breakpoint model), or a quadratic or second order polynomial regression, is the most appropriate statistical method to apply to requirement studies (Baker, 1986). A continuous broken line

calculated by least squares is the preferred method because it objectively selects the breakpoint in the response line (Baker, 1986). For that reason our research group selected and implemented the continuous broken line model (two-phase linear crossover model) to interpret amino acid requirements. This analysis iteratively partitions the individual data points between two lines and selects the breakpoint or requirement of the test amino acid that minimizes the unexplained variation, as calculated by least squares (Seber, 1977). The two-phase linear crossover model clearly defines an objective breakpoint and this breakpoint represents the mean requirement of the population. The error terms for the lines also allows calculation of the confidence intervals of the requirement, or more importantly the upper 95% interval or the safe level of intake, meeting the requirement of 95% of the population. The FAO/WHO/UNO (1985) uses the upper 95% confidence interval in setting the dietary recommendation for amino acids for humans. In contrast, the Nutrient Requirements for Swine (NRC, 1998) publishes the population mean requirement and allows nutritionists to select their own reasonable safety factor to cover the differences among diets, animals and environments.

To properly assess the data produced from a requirement study, the expression of the data must also be considered. The nutrient requirements of animals are generally expressed as % of diet, g/d, g/MJ or g/kcal and g $kg^{-1}d^{-1}$. The first, second and third terms (% of diet, g/d and g/MJ or g/kcal) require feed intake to also be reported in order to determine the actual intake of the test nutrient. Representing nutrient requirements per unit of body weight is preferable and accounts for intake and differences in body weight. We have chosen to present requirements on a g kg^{-1} d^{-1} basis to avoid the complication of heavier animals requiring greater amounts of the nutrient (g d^{-1}). Also commonly used in

the presentation of an amino acid requirement is g / 100 kcal metabolizable energy (Baker, 1986) and we provide the reader with the energy density of the diet if this method is preferable.

The digestibility, effect on endogenous secretions and true availability of an amino acid also must be considered. Amino acids requirements for pigs can be represented on an ileal digestible, true ileal digestible or more recently on a true metabolic availability. Ileal digestibilities are a crude measure of small intestinal absorption of an amino acid, while true ileal digestibilities correct for endogenous secretions. Last, true availability is a measure of the metabolic availability of the amino acid for protein synthesis; thus, accounting for differences in digestibility and metabolic differences. For example, our research group has recently shown that the threonine requirement in grower pigs is higher when they are fed a barley diet vs. a casein diet (Myrie et al, MSc thesis, 200) due to the higher fibre content of the barley diet. To avoid the complication due to the lack of knowledge of true availability, we supply the same elemental solution parenterally and enterally during requirement studies and assume that the free amino acids supplied by the solution are 100% bioavailable. However, knowing the bioavailability of methionine and cysteine in diet ingredients is especially important when attempting to define the ability of cysteine to spare the methionine requirement (Chung and Baker, 1992).

Differences in rates of deposition of protein among growing animals may also affect the estimate of the requirement, as leaner growing animals will have a greater requirement for amino acids as a percentage of the diet (Baker, 1986). However, differences in the rate of protein deposition between sexes do not occur prior to the

initiation of puberty and does not need to be considered for studies examining neonates. In addition, by utilizing the same breed of pig for our studies, we should not have differences in rates of protein deposition.

Presently, the most frequent criticism in the design of indicator amino acid oxidation studies is that data is collected only in the fed state following a short period of adaptation to a new dietary level of the test amino acid. This adaptation issue arose because nitrogen balance studies require a long adaptation period (7-10 days) to stabilize urinary nitrogen excretion and thus it came to be believed that all aspects of amino acid metabolism required a long adaptation period to adapt to dietary changes. However, changes in dietary amino acid intake changes the aminoacyl t-RNA levels in less than 4 hours (Crim and Munro, 1994). Furthermore, the oxidation of an indicator amino acid changes and stabilizes within 4 hours of changes in dietary amino acid intake in adult humans (Zello et al, 1995, Pencharz and Ball, 2003) and oxidation rate is similar 16 hours and 10 days after a dietary change in amino acids (Moehn et al, 2004). The risks associated with longer adaptation must also be considered. Too long an adaptation period will deplete the whole body pool of the test amino acid, which may cause further metabolic changes that could affect the estimate of the requirement. Therefore, we believe that a short adaptation period should be used because it results in sensitive and reproducible changes in amino acid oxidation. The second issue is whether oxidation studies can be conducted only in the fed state or whether both fed and fasted periods need to be measured. Although amino acid oxidation is higher in the fed state, calculation of the lysine requirement in human adults was not different when 24h fed and fasted was compared to fed only data (Kurpad et al, 2001); therefore, measuring requirements in

both fed and fasted states or the fed state are appropriate. The rapid change in oxidation response to a change in intake of a limiting amino acid is due to the lack of a storage compartment for free amino acids and the short half life of the plasma free amino acid pool.

Therefore, there are numerous considerations when designing a requirement study. At least three graded dietary levels are required, preferably six or more, to define the response to the test nutrient. The subsequent statistical analysis should define the mean requirement and upper 95% confidence interval for safe intake. Components of the diet that may affect the nutrient bioavailability, such as fibre, also need to be considered. The variability between subjects can be affected by body composition, so sex differences, and in the case of pigs, breed differences, can significantly affect the estimate of an amino acid requirement. Last, although changes in amino acid content will change rapidly, the protein content of the diet should be standardized prior to initiation of test diets. Ultimately, by properly designing requirement studies, any bias can be removed and with proper analysis of the data, an objective estimate of the requirement can be made.

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2.0 RATIONALE AND OBJECTIVES

2.1 Scope of thesis

The aim of the research within this thesis is to examine the role of the splanchnic tissues in sulfur amino acid metabolism. From this point forth, we will assume that the majority of differences in sulfur amino acid metabolism between enteral and parenteral feeding are due mainly to by-pass of intestinal metabolism. To achieve this, specific hypotheses and objectives were developed and are summarized below.

2.2 Rationale

Recent research has suggested that the gut is utilizing indispensable amino acids; however, the metabolic fate of these amino acids remains unclear. Understanding the role of the gut is important in situations where the gut is compromised due to disease or stress; such as that which commonly occurs during weaning. This knowledge is also important when feeding animals parenterally, because first pass gut and liver metabolism is by-passed. The majority of research examining the sulfur amino acids has focused on hepatic metabolism, while the capacity of the gut for the utilization and degradation of the sulfur amino acids and the synthesis of cysteine is not understood. Recent evidence (Stoll et al, 1998) has suggested that the gut is responsible for utilization of ~50% of dietary methionine, suggesting that the total sulfur amino acid requirement of the pig may be lower when the gut is by-passed. Previous research has suggested that cysteine is an indispensable amino acid during parenteral feeding and may also be indispensable in the neonate; however, further research is necessary to establish why plasma cysteine concentrations are low when the gut is bypassed during parenteral feeding. The

investigations in this thesis will ultimately lead to a better understanding of sulfur amino acid metabolism in both enterally and parenterally fed neonates. These investigations should lead to improvements in the formulation of diets for early-weaned piglets, populations with gut stress or disease and formulation of parenteral solutions for human neonates.

2.3 Specific hypotheses and objectives

Hypothesis 1: The methionine requirement is significantly lower during parenteral versus enteral feeding in the neonatal piglet.

This first study in this thesis will measure the methionine requirement, with no cysteine, during both parenteral and enteral feeding in the neonatal piglet by employing the indicator amino acid oxidation technique. If cysteine is not a conditionally indispensable amino acid, then the methionine requirement can be considered to be the requirement for the total sulfur amino acids. To directly compare the parenteral and enteral requirements, a similar elemental diet will be fed intragastrically and intravenously. Comparison of the methionine requirement between routes of feeding will allow an estimate of the utilization of the sulfur amino acids by the splanchnic tissues.

Hypothesis 2a: The methionine requirement, in the presence of excess cysteine, is lower during parenteral versus enteral feeding, in the neonatal piglet. **Hypothesis 2b:** Cysteine can spare the methionine requirement by approximately 50% in both routes of feeding. **Hypothesis 2c:** Cysteine is a conditionally indispensable amino acid during parenteral or enteral feeding in the neonatal piglet.

This second study in this thesis will directly measure the methionine requirement, in the presence of excess cysteine, during both parenteral and enteral feeding in the neonatal piglet by employing the indicator amino acid oxidation technique (Hypothesis 2a). Comparison of the parenteral and enteral methionine requirements, with provision of excess cysteine, will allow an additional estimate of the utilization of the sulfur amino acids by the splanchnic tissues, similar to hypothesis 1.

Subsequent comparison of this study to the first study will allow a calculation of the cysteine sparing capacity during both parenteral and enteral feeding (Hypothesis 2b). Furthermore, comparison between the baseline oxidation (after the requirement for methionine has been met) in the first study with that of the second study will allow an estimate of whether cysteine is a conditionally indispensable amino acid for protein synthesis in the neonate receiving either parenteral nutrition or enteral nutrition (Hypothesis 2c).

Hypothesis 3: Plasma homocysteine concentrations are positively associated to methionine intake in both parenterally and enterally fed piglets.

No studies examining the effects of the dietary ratio of methionine: cysteine and route of feeding on plasma homocysteine concentrations in neonatal animals were found in the literature. Therefore, plasma homocysteine concentrations were measured in samples from the previous two studies. Because it is well accepted that the liver is the main site of sulfur amino acid metabolism, the dietary intakes of dietary sulfur amino acids should be positively correlated to plasma homocysteine in both routes of feeding and whether dietary cysteine was provided or not. Furthermore, because dietary cysteine has been shown to reduce transmethylation and increase remethylation, the addition of

dietary cysteine to both parenteral and enteral diets should produce lower plasma total homocysteine concentrations compared to diets that provided methionine alone.

Hypothesis 4: N-acetyl-L-cysteine is a bioavailable precursor for L-cysteine in neonatal piglets receiving parenteral nutrition.

Plasma concentrations of cysteine during parenteral feeding are low compared to enteral feeding and this indicates that cysteine supplementation may be required during parenteral feeding to maintain cysteine availability to the peripheral tissues. However, L-cysteine is highly unstable in solution; therefore, a stable precursor for cysteine must be identified before cysteine supplementation to parenteral solutions will occur on a widespread basis. The bioavailability of a potential precursor of L-cysteine, N-acetyl-L-cysteine will be assessed using the intravenously fed neonatal piglet model. A variety of metabolic parameters will be measured as indicators of the bioavailability of N-acetyl-L-cysteine.

Hypothesis 5: Cysteine synthesis (methionine oxidation) is lower in the parenterally fed compared to the enterally fed neonatal piglet and cysteine oxidation is similar whether total nutrition is parenterally or enterally delivered.

This final experiment will assess the differences in cysteine synthesis, as measured by methionine oxidation, and in cysteine oxidation, in both parenterally and enterally fed neonatal piglets. The route of tracer administration will be the same as diet administration; therefore, the difference in methionine and cysteine oxidation between enteral and parenteral administration will determine the degree to which the gut is responsible for cysteine synthesis and/or cysteine catabolism. This experiment will also determine, using a different isotopic tracer to examine the cysteine sparing mechanism,

whether cysteine can spare the methionine requirement (Hypothesis 2b). This will be achieved by keeping methionine constant at ~ 50% of total sulfur amino acid requirement and increasing the cysteine intake from 0 to 100% of total sulfur amino acid requirement. Cysteine sparing will be confirmed if methionine oxidation is reduced in response to increasing cysteine intake.

3.0 EXPERIMENTAL SECTION

3.1 THE METHIONINE REQUIREMENT IS LOWER IN PARENTERALLY FED NEONATAL PIGLETS THAN IN ENTERALLY FED.

3.1.1 Introduction

Due to immaturity and congenital abnormalities, neonates frequently require parenteral feeding (Ball et al, 1996). Part of the goal of parenteral feeding is to provide essential and non-essential nutrients at a level sufficient to meet maintenance and growth requirements while avoiding an excessive supply, which may put undue stress on these neonates' immature organ systems (Ball et al, 1996, Pencharz et al, 1996). Presently, the amino acid profile of most parenteral solutions is based upon reference proteins such as egg protein or human breast milk (Brunton et al, 2000). This may not be the correct profile when the gut is atrophied and has a lower metabolic demand (Brunton et al, 2000). Wykes et al (1992) demonstrated significant differences in amino acid kinetics (flux, oxidation, synthesis (non-oxidative disposal), and release from protein) between oral and parenteral feeding in the neonatal infant. We demonstrated recently that the threonine requirement is 55% lower (Bertolo et al, 1998) and the branched chain amino acid requirement is approximately 44% lower (Elango et al, 2002) in neonatal piglets fed parenterally than in those fed enterally. In addition, we have measured the tryptophan requirement in parenterally and enterally fed neonatal piglets and found that it did not differ between the two groups (Cvitkovic et al, *unpublished*). This suggests that some amino acids are completely absorbed into the portal vein and not metabolized by the splanchnic tissues, that differences in the requirement cannot be detected or that the

source of amino acids for intestinal protein synthesis is different. Although the reduced intestinal protein synthesis that is associated with parenteral feeding may account for a proportion of these reductions in amino acid requirements, this alone is not sufficient to account for the observed differences in parenteral and enteral amino acid requirements. Therefore, both parenteral and enteral amino acid requirements need to be determined empirically to ensure that they are supplied in an optimum profile.

No reports were found in the literature where the methionine requirement was determined directly in piglets fed intravenously. Most studies in the parenterally fed neonate have examined the possibility of the essentiality of cysteine and taurine but not the requirement for methionine (Pascal et al, 1972, Gaull et al, 1972, Pohlandt et al, 1974, Helms et al, 1995). However, there are studies that have determined the methionine requirement in orally fed growing (> 5kg body weight) and mature animals by measuring growth, nitrogen balance, plasma free amino acids, plasma or blood urea nitrogen (Reifsnyder et al, 1984, Balogun and Fetuga, 1981). Other researchers have used the ileal digestibility assay (Chung and Baker, 1992) and to our knowledge, only one researcher has used an isotopic technique to measure the sulfur amino acid requirement in young pigs (Kim and Bayley, 1983a). Because there is no evidence that cysteine synthesis is limited in neonatal piglets, in the present study methionine only was used to supply total sulfur amino acids.

Classical methods of determining indispensable amino acid requirements, such as nitrogen balance and the direct tracer approach, require prolonged adaptation and are therefore unsuitable for use in the human neonate (Brunton et al, 1998, Brunton et al, 2000). For this reason, we developed a piglet model for the human neonate (Wykes et al,

1993). We have also validated the novel and minimally invasive technique of indicator amino acid oxidation as a means of determining indispensable amino acid requirements in piglets fed enterally or parenterally (Bertolo et al, 1998 and Brunton et al, 2000). In subsequent studies in human neonates, we showed that findings in the piglet model are valid in parenterally fed human infants (Roberts et al, 2001). In the present study, the objective was to determine the methionine requirement in a neonatal piglet model receiving parenteral (TPN) (n=18) or enteral nutrition (n=14) by employing the indicator amino acid oxidation technique using L-[1-^{14}C]phenylalanine. We hypothesized that the parenteral methionine requirement would be significantly lower than the enteral requirement.

3.1.2 Materials and Methods

3.1.2.1 *Animals and study protocol.*

The Animal Care Committee at the University of Alberta approved all procedures in this study. A total of 25 male and 7 female Landrace/Large White piglets (Genex Swine Group) were obtained from the University of Alberta, Swine Unit (Edmonton, AB, CAN) at a mean age of 1.7 d of age and a mean weight of 1.66 kg. Piglets were transported to the Metabolic Unit at the University of Alberta. The piglets were weighed and then anaesthetized with acepromazine (0.5 mg/kg; Atravet™; Ayerst Laboratories, Montreal, PQ) and ketamine hydrochloride (22 mg/kg; Rogarsetic™; Rogar STB Inc., Montreal, PQ) and maintained during surgery with 1% halothane. All piglets were then fitted with venous catheters (Ed-Art, Don Mills, Canada) using the modified methods of Wykes et al. (1993); in enterally fed piglets, gastric catheters were inserted using the

method of Rombeau et al. (1984). In all pigs, an infusion catheter was inserted into the left jugular vein and advanced to the superior vena cava just cranial to the heart and a sampling catheter was inserted into the left femoral vein and advanced to the inferior vena cava just caudal to the heart. After surgery, incision sites were treated with a topical antibiotic (Hibitane Veterinary Ointment: Ayerst Laboratories, Montreal, PQ), and an analgesic (0.1 mg/kg Buprenex, Buprenorphrine HCl, Reckitt and Colman Pharmaceutical Inc., Richmond, VA) was given intramuscularly. Analgesic was given again 8 and 16 h post-surgery. Piglets were then put into cotton jackets, which secure the tether to the piglets. The tether is part of the swivel- tether system (Alice King Chatham Medical Arts, Los Angeles, California), which enables the piglets to move freely while receiving a continuous dietary infusion and ensuring that the catheters to do not become tangled and occluded.

3.1.2.2 ***Animal housing.***

Animals were kept in rooms between 21°C-27°C, with supplemental heat from heat lamps provided for each individual cage. Lighting was on a 12 h light: dark schedule. Each piglet was placed in an individual circular cage, 75 cm in diameter with toys to enhance their environment.

3.1.2.3 ***Diet regimen.***

Elemental solutions were designed to meet the requirements of neonatal piglets (Wykes et al, 1993). Diets were administered using pressure-sensitive infusion pumps. Piglets received 15 g amino acids $kg^{-1} \cdot d^{-1}$ and 1.1 MJ metabolizable energy $kg^{-1} \cdot d^{-1}$ with glucose and lipid (Intralipid 20%, PharmaciaUpjohn, Stockholm, Sweden) each supplying 50% of nonprotein energy intake. The base amino acid profile of the complete

elemental diet fed from d 0 to 5 is described in **Table 3.1.1**. The amino acid profile was based on human milk protein (Vaminolact: Fresenius-Kabi) except phenylalanine and tyrosine which were provided at their estimated safe levels of intake (House et al, 1997a, House et al, 1997b) and arginine provided at 1.2 g kg^{-1} d^{-1} (Brunton et al, 1999). Tyrosine was provided as the dipeptide glycyl-tyrosine (House et al, 1997b). Full infusion rates (272 mL kg^{-1} d^{-1}) were adjusted on a weight basis so that energy and nitrogen intake was identical for all piglets. All vitamins were supplied in a commercial solution, MVI Paediatric (Rhone-Poulenc Rorer Canada Inc, Montreal, PQ), which provides a combination of oil and water-soluble vitamins, formulated especially for incorporation into intravenous solutions. The cofactors involved in the transsulfuration pathway, vitamin B-12, choline, B-6 and folate, were in the MVI solution at approximately 115% of requirement (NRC, 1998). Piglets also received a mineral solution including zinc, copper, manganese, chromium, selenium and iodide at 200% of the NRC (1998) recommendation for piglets.

All piglets received a amino acid and glucsoe/lipid (5:1) solution intravenously at 50% infusion rate (6.75 mL kg^{-1} h^{-1}) for 12 hours beginning immediately following surgery and then 75% infusion rate (10.125 mL kg^{-1} h^{-1}) until the morning after surgery (d 1). Intravenously fed piglets then received TPN and lipid intravenously at 13.5 mL kg^{-1} h^{-1} for the remainder of the study. Enterally fed pigs received TPN and lipid intragastrically at 6.75 mL kg^{-1} h^{-1} for 8h, 10 mL kg^{-1} h^{-1} for 8 h and then 13.5 mL kg^{-1} h^{-1} for the remainder of the study. All piglets received complete TPN and lipid until 1800 h on d 5. Piglets were then randomly allocated to one of seven test levels of methionine

Table 3.1.1: Amino acid concentration of parenteral and enteral solutions administered to neonatal piglets in enterally and parenterally fed piglets from d0 to d5.

Amino acid	*Concentration (g L^{-1})*
L-Alanine	5.88
L-Arginine	4.41
L-Aspartate	3.34
L-Cysteine	0.80
L-Glutamate	5.77
Glycine	1.36
L-Histidine	1.72
L-Isoleucine	2.53
L-Leucine	5.77
L-Lysine [1]	5.73
L-Methionine	1.06
L-Phenylalanine	1.76
L-Proline	4.54
L-Serine	3.06
Taurine	0.24
L-Threonine	2.91
L-Tryptophan	1.17
L-Tyrosine	1.50
L-Valine	2.94
Glycyl-Tyrosine[2]	1.43

[1] Lysine was supplied as Lysine-HCl to provide the concentration shown.
[2] Glycyl tyrosine supplying 0.45 g L^{-1} of glycine and 1.09 g L^{-1} tyrosine.

Parenterally fed: 0.05, 0.1, 0.2, 0.3, 0.4, 0.6 and 1.0 g kg^{-1} d^{-1}; Enterally fed: 0.1, 0.2, 0.4, 0.5, 0.6, 0.7, 1.0 g kg^{-1} d^{-1}) with no cysteine included. Therefore, these methionine intakes ranged from 3.3 to 70 mg/g and 6.7 to 70 mg/g of total amino acids for parenterally and enterally fed piglets, respectively. All test diet solutions were made isonitrogenous by dissolving the appropriate quantity of L-methionine (at test level) and L-alanine (to maintain isonitrogenous solutions) in 75 mL distilled water; these solutions were then transferred into infusion bags containing 675 mL of concentrated TPN solution without any L-methionine or L-alanine. The solutions were filtered with a 0.22-μm filter (Millipore, Milford, MA). Piglets were administered the test diet from 1800h on d 5 until the completion of the oxidation study on d 6. After the d 6 oxidation study, piglets were returned to the complete diet for 24 h. At 1800h on d 7, piglets were randomly assigned to a second test diet and a second oxidation study was performed on d 8. Previously, we have determined that a second indicator amino acid oxidation study 48 h after the first study produces data not different from the first study; therefore two studies can be conducted to reduce the number of animals required for an entire indicator amino acid oxidation requirement study (Brunton and Ball, *unpublished data).*

3.1.2.4 ***Tracer infusion, $^{14}CO_2$ collection and analytical procedures.***

Phenylalanine oxidation and flux were determined by a primed (186 kBq (5 μCi/kg), constant intravenous infusion (130 kBq (3.5 μCi kg^{-1} h^{-1})) of a tracer solution containing 92.8 MBq (2.5 mCi)/L of L-[1-^{14}C]phenylalanine (200 MBq (54 mCi/mmol; American Radiolabeled Chemicals, Inc. St. Louis, MO). To reach plateau in both breath and blood labeling, the duration of constant infusion was 4h. Details of infusion protocol, $^{14}CO_2$ collection and blood collection procedures have been described

previously (House et al, 1997a). Following the infusion on d 8, piglets were killed by injection of 1000 mg of sodium pentobarbital into a venous catheter.

Reverse-phase high performance liquid chromatography with the use of phenylisothiocyanate derivatives was used to measure plasma amino acids. Collection and liquid scintillation counting of radioactive fractions to determine specific radioactivity (SRA) of plasma phenylalanine and tyrosine were completed as previously described (House et al, 1997a). The calculations for intake, oxidation, flux, non-oxidative disposal, release from protein breakdown, balance and percent dose oxidized were as reported previously (House et al, 1997a). SRA for both plasma phenylalanine and tyrosine during the IAAO study were plotted and plateau values were calculated as the mean SRA at plateau. Plasma concentrations are represented as the mean concentration at each test methionine level.

3.1.2.5 ***Statistical analyses.***

A completely randomized design was used, with methionine intake serving as the main treatment effect. The effects of day of indicator amino acid oxidation study (d 6 or d 8), gender of pig, initial weight, and weight at study were determined not to be significant using an ANOVA (SAS/STAT, version 8.1, SAS Institute, Cary, NC). If P values were < 0.05 for the F-value of the ANOVA model, significant differences between treatments were assessed using Student Newman Keul's multiple comparison procedure. Determination of methionine requirement was performed using a two-way linear crossover model, as described previously (Seber, 1977 and Ball and Bayley, 1984). Regression analysis variables were dietary concentration of methionine as the independent variable and percentage of dose oxidized and phenylalanine oxidation as the

dependent variable. The 95% confidence intervals, for the estimation of a safe level of intake, were also determined.

To compare the breakpoints or requirement estimates between parenterally and enterally fed piglets, we treated the breakpoint as a sample mean and used the pooled two-sample *t* procedure (Pagano and Gavreau, 2000). Based on the assumption that the subjects are derived from the same population and identical procedures were used, the true variance was assumed to be the same. The pooled variance was calculated by averaging each sample variance with weights equal to its degrees of freedom. Therefore, pooled variance for the two groups was determined and used to test if the two breakpoints were different using a pooled two-sample *t* procedure.

3.1.3 **Results**

All piglets were healthy and active during the course of the study. Piglet weight upon arrival (1.66 kg, pooled SD= 0.15) and weight at study (2.71 kg, pooled SD= 0.32) did not differ among diet levels or route of feeding. Rates of average daily gain for the 5 day adaptation period prior to test diet initiation were not significantly different (156 g/d for parenterally and 181 g/d enterally fed piglets).

3.1.3.1 ***Parenteral and enteral methionine requirement.***

Values for $^{14}CO_2$ recovery and plasma SRA for both parenteral and enteral requirements are summarized in **Tables 3.1.2** and **Table 3.1.3,** respectively. Plateaus in breath $^{14}CO_2$, plasma phenylalanine SRA and plasma tyrosine SRA were reached by 2 h after the initiation of the primed, constant infusion in all pigs. The ratio of plasma

Table 3.1.2: Plateau values for $^{14}CO_2$ recovery and phenylalanine (Phe) and tyrosine (Tyr) specific radioactivity (SRA) in plasma of piglets receiving total parenteral nutrition with graded levels of methionine[1]

	Methionine Intake (g kg^{-1} d^{-1})								
	0.05	0.1	0.2	0.3	0.4	0.6	1.0	pooled	ANOVA
n	5	6	4	5	4	4	4	SE	P value
Percent of phenylalanine oxidized[2]	7.0^{a}	3.4^{ab}	2.2^{b}	3.3^{ab}	0.92^{b}	0.57^{b}	0.31^{b}	0.56	0.005
Expired $^{14}CO_2$, (x 10^3 volume corrected), DPM kg^{-1} h^{-1},[4]	421.4	250.9	151.9	218.3	87.4	78.5	21.5	38.2	NS[3]
Phe SRA, (x 10^3 DPM $umol^{-1}$)	34.3	46.9	20.3	24.3	28.4	16.5	28.4	5.8	NS
Tyr SRA (x 10^3 DPM $umol^{-1}$)	3.0	2.7	2.6	3.3	2.5	0.5	0.6	0.3	NS
Tyr SRA: Phe SRA	12.5	12.9	11.9	14.2	8.2	4.6	2.7	2.0	NS

[1]Values represent the means of the number of pigs indicated at the top of each column
[2]Overall ANOVA, F-test, $P<0.01$. Values with different superscript letters indicate a significant difference among diet levels (Student Newman Keul's' multiple comparisons).
[3] Non-significant is abbreviated to NS.
[4] Values corrected for a 93.3 % bicarbonate retention factor.

Table 3.1.3: Plateau values for $^{14}CO_2$ recovery and phenylalanine (Phe) and tyrosine (Tyr) specific radioactivity (SRA) in plasma of piglets receiving total nutrition intragastrically with graded levels of methionine[1]

	Methionine Intake (g kg^{-1} d^{-1})								
	0.1	0.2	0.4	0.5	0.6	0.7	1.0	pooled	ANOVA
n	4	3	4	4	4	3	4	SE	P value
percent of phenylalanine oxidized[2]	5.97[a]	4.28[a]	0.87[b]	0.45[b]	0.34[b]	0.44[b]	0.50[b]	0.47	<0.0001
Expired $^{14}CO_2$, (x 10^3 volume corrected), DPM kg^{-1} h^{-1},[4]	391[a]	296[a]	58[b]	31[b]	23[b]	29[b]	36[b]	28	<0.0001
Phe SRA, (x 10^3 DPM $umol^{-1}$)	59.1	36.0	29.3	32.2	49.2	25.1	63.5	4.6	NS[3]
Tyr SRA (x 10^3 DPM $umol^{-1}$)	2.5	2.0	0.8	1.5	1.8	0.9	2.1	2.0	NS
Tyr SRA: Phe SRA	5.6	5.2	2.6	4.5	3.2	4.1	4.0	0.46	NS

[1]Values represent the means of the number of pigs indicated at the top of each column
[2]Overall ANOVA, F-test, P<0.01. Values with different superscript letters indicate a significant difference among diet levels (Student Newman Keul's' multiple comparisons).
[3] Non-significant is abbreviated to NS.
[4] Values corrected for a 93.3 % bicarbonate retention factor.

tyrosine SRA/ plasma phenylalanine SRA was not significantly different among diet treatments; thus, the rate of conversion of phenylalanine to tyrosine did not affect the breakpoint estimate.

3.1.3.2 ***Parenteral methionine requirement.***

Phenylalanine flux, intake, non-oxidative disposal and release from protein were not significantly different ($P>0.05$) among dietary treatments (**Table 3.1.4**). The similarity in flux among dietary treatments indicates that the differences observed in phenylalanine oxidation reflect a shift in partitioning between oxidation and protein synthesis. Furthermore, the lack of difference in tyrosine: phenylalanine SRA demonstrates that when dietary tyrosine is in excess, phenylalanine is channeled directly to oxidation within the hepatocyte and is partitioned between protein synthesis and oxidation (House et al, 1997a and Shinman and Gray, 1988). Phenylalanine oxidation, expressed as percent of the dose oxidized and as the absolute rate, was significantly influenced by methionine intake ($P= 0.005$ and $P<0.0001$, respectively (**Figure 3.1.1**). As methionine intake increased from 0.05 g kg-1 d-1 to 0.29 g kg-1 d-1, phenylalanine oxidation (% of dose) decreased ($P<0.05$). Further increases in methionine intake did not affect phenylalanine oxidation ($P>0.05$, slope not different from zero) (Figure 1).

To determine the methionine requirements, the data points were partitioned between two distinct regression lines (Figures 3.1.1 and **Figure 3.1.2**). The final regression was chosen as the model that produced the highest regression coefficients for the dependent variables. The breakpoint is the point at which the two regression lines intersected and gives an estimate of the mean methionine requirement; from the variation

Table 3.1.4: Phenylalanine kinetics (μmol kg^{-1} h^{-1}) of piglets receiving total parenteral nutrition with graded levels of methionine[1]

	Methionine Intake (g kg^{-1} d^{-1})								
	0.05	0.1	0.2	0.3	0.4	0.6	1.0	pooled	ANOVA
n	3	5	3	4	4	2	4	SE	P value
(μmol kg^{-1} h^{-1})									
Flux	425	254	303	257	256	342	274	18.9	NS[3]
Intake	104	112	110	119	109	121	105	2.5	NS
Oxidation[2]	38^a	9^b	17^b	4^b	3^b	5^b	0.7	2.5	<0.0001
Non-Oxidative Disposal	387	246	297	253	254	338	273	17.6	NS
Release from protein	330	142	188	140	148	212	169	19.2	NS

[1]Values represent the means of the number of pigs indicated at the top of each column
[2]Overall ANOVA, F-test, P<0.01. Values with different superscript letters indicate a significant difference among diet levels (Student Newman Keul's' multiple comparisons).
[3] Non-significant is abbreviated to NS.

Figure 3.1.1: ***Parenteral methionine requirement:*** **oxidation of L-[1-^{14}C]phenylalanine as a percentage of dose in parenterally fed piglets receiving graded levels of methionine (n=32)**

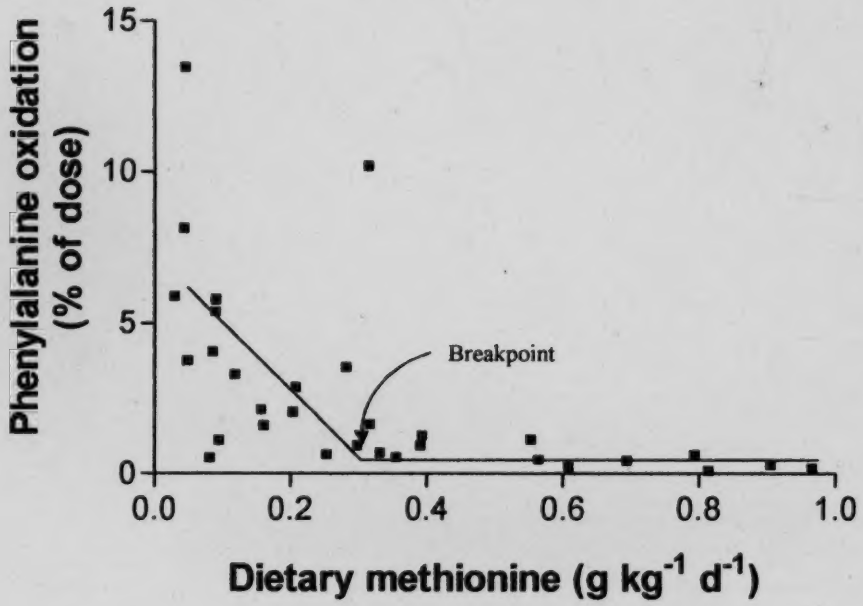
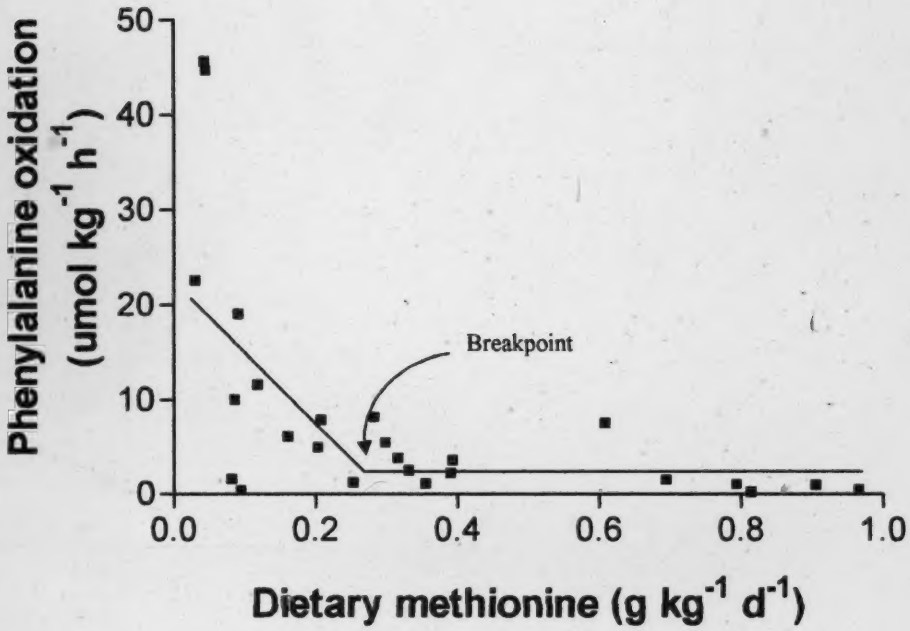


Figure 3.1.2: ***Parenteral methionine requirement:*** **rate of L-[1-^{14}C]phenylalanine oxidation (μmol/(kg h)) in parenterally fed piglets receiving graded levels of methionine (n=32)**



associated with the regression lines, the corresponding confidence intervals are calculated. The breakpoint estimate for phenylalanine oxidation rate (Figure 3.1.2) (requirement = 0.26 g kg^{-1} d^{-1}, 95% CI: 0.08-0.43 g kg^{-1} d^{-1}) was similar to that for phenylalanine oxidation rate as a percentage of dose (Figure 3.1.1) (requirement = 0.29 g kg^{-1} d^{-1}, 95% CI: 0.15-0.42 g kg^{-1} d^{-1}). Methionine intake did not influence the plasma free amino acid concentrations of any amino acids ($P>0.05$) in particular the sulfur amino acids, methionine, and cystine (**Table 3.1.5**). However, in contrast to normal concentrations of plasma cysteine ≈50 μmol/L (Bertolo et al, 2000), these were very low.

3.1.3.3 ***Enteral methionine requirement.***

Phenylalanine flux, intake , non-oxidative disposal and release from protein were not significantly different ($P>0.05$) among dietary treatments (**Table 3.1.6**). Phenylalanine oxidation, expressed as percent of the dose oxidized and as an absolute rate was significantly influenced by methionine intake ($P<0.005$). As methionine intake increased from 0.1 g kg^{-1} d^{-1} to 0.42 g kg^{-1} d^{-1}, phenylalanine oxidation (% of dose) decreased ($P<0.05$) (**Figure 3.1.3**). Further increases in methionine intake did not effect phenylalanine oxidation ($P>0.05$, slope not different from zero) (Figure 3).
The breakpoint estimate for phenylalanine oxidation rate (**Figure 3.1.4**) (requirement = 0.44 g kg^{-1} d^{-1}, 95% CI: 0.31-0.58 g kg^{-1} d^{-1}) was similar to that for phenylalanine oxidation rate as a percentage of dose (Figure 3.1.3) (requirement = 0.42 g kg^{-1} d^{-1}, 95% CI: 0.35-0.48 g kg^{-1} d^{-1}). Similarly to the parenterally fed piglets, dietary treatment did not affect the plasma concentration of the sulfur amino acids. Increasing methionine intake resulted in a decrease in plasma glutamine, phenylalanine and tyrosine

Table 3.1.5: ***Parenteral methionine requirement*: plasma amino acid concentrations (umol/L) of piglets receiving total parenteral nutrition with graded levels of methionine[1]**

	Methionine Intake (g kg^{-1} d^{-1})								
amino acid (umol L^{-1})	0.05	0.1	0.2	0.3	0.4	0.6	1.0	pooled SE	ANOVA P value
n	3	5	3	4	4	2	4		
cystine	28	28	25	23	25	56	26	3	NS[3]
glutamine	342	321	220	246	148	237	237	23	NS
methionine	23	18	19	25	15	27	25	2	NS
phenylalanine	68	72	53	66	45	98	60	4	NS
taurine	173	135	114	109	80	103	180	12	NS
tyrosine	218	200	96	120	56	125	177	23	NS

[1]Values represent the means of the number of pigs indicated at the top of each column.
[3] Non-significant is abbreviated to NS.

Table 3.1.6: *Enteral methionine requirement*: phenylalanine kinetics (μmol kg^{-1} h^{-1}) of piglets receiving total nutrition intragastrically with graded levels of methionine[1]

	Methionine Intake (g kg^{-1} d^{-1})								
	0.1	0.2	0.4	0.5	0.6	0.7	1.0	pooled	ANOVA
n	4	3	4	4	4	3	4	SE	P value
(μmol kg^{-1} h^{-1})									
Flux	163	195	252	236	148	295	137	17	NS[3]
Intake	118	118	114	118	113	120	116	1	NS
Oxidation[2]	19.9^{a}	8.9^{ab}	2.3^{bc}	1.1^{c}	0.5^{c}	1.2^{b}	0.7^{c}	0.9	0.0008
Non-Oxidative Disposal	153	186	250	234	147	294	136	17	NS
Release from protein	44	76	137	117	34	174	20	17	NS

[1]Values represent the means of the number of pigs indicated at the top of each column
[2]Overall ANOVA, F-test, $P<0.01$. Values with different superscript letters indicate a significant difference between diet levels (Student Newman Keul's multiple comparisons).
[3] Non-significant is abbreviated to NS.

Figure 3.1.3: ***Enteral methionine requirement:*** **oxidation of L-[1-^{14}C]phenylalanine as a percentage of dose in enterally fed piglets receiving graded levels of methionine (n=32)**

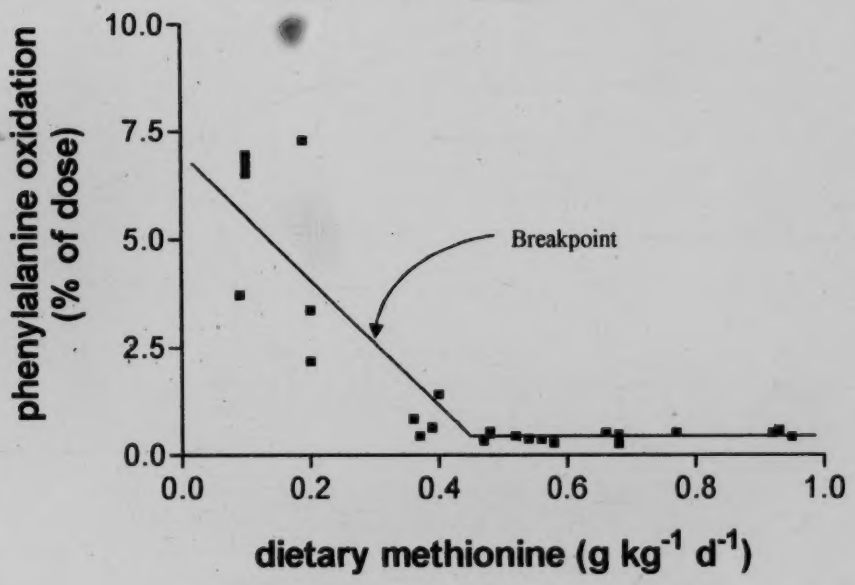
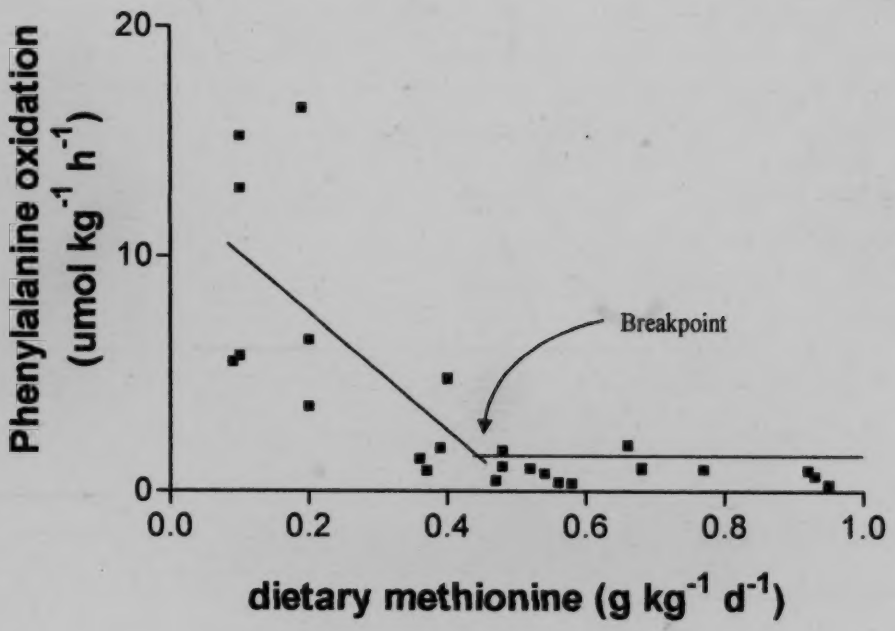


Figure 3.1.4: ***Enteral methionine requirement:*** **rate of L-[1-^{14}C]phenylalanine oxidation (μmol/(kg h)) in parenterally fed piglets receiving graded levels of methionine (n=32)**



(P<0.05); all other plasma amino acids did not demonstrate any significant change among diet levels (**Table 3.1.7**).

The comparison between means (breakpoint or requirement) of parenterally and enterally fed piglets resulted in a significant difference (P<0.005). This difference indicates that the intravenous requirement is 69% of the enteral requirement.

3.1.4 **Discussion**

The neonatal piglet model, in combination with the indicator amino acid oxidation technique, has been successfully applied to determine the parenteral lysine (House et al, 1998), phenylalanine (House et al, 1997a), parenteral and enteral threonine (Bertolo et al, 1998), and branched chain amino acid (Elango et al, 2002) requirements. Because methionine has a complex pathway with multiple fates, the direct amino acid oxidation technique is difficult to apply and interpret because CO_2 is only released when S-adenosylmethionine is decarboxylated to decarboxylated-adenosylmethionine or by the degradation of α-ketobutyrate to propionyl CoA. To the authors' knowledge the present report is the first direct measurement of parenteral requirements for the sulfur amino acids in a neonatal model under well-controlled conditions. Comparison of the parenteral and enteral requirement also provides an estimation of the extent of first pass metabolism of the sulfur amino acids by the splanchnic tissues.

The mean parenteral methionine requirement, as determined by a two-phase linear crossover model, was 0.29 g kg^{-1} d^{-1} when based on phenylalanine oxidation as a percentage of the dose oxidized, and 0.26 g kg^{-1} d^{-1} when based on phenylalanine

Table 3.1.7:*Enteral methionine requirement*: plasma amino acid concentrations (umol/L) of piglets receiving total nutrition intragastrically with graded levels of methionine[1]

	Methionine Intake ($g\ kg^{-1}\ d^{-1}$)								
amino acid ($umol\ L^{-1}$)	0.1	0.2	0.4	0.5	0.6	0.7	1.0	pooled SE	p value
n	4	3	4	4	4	3	4		-
cystine	59	57	77	86	90	83	79	5	NS[3]
glutamine[2]	348[a]	269[ab]	150[ab]	128[ab]	128[ab]	106[b]	144[ab]	22	0.010
methionine	41	36	45	39	38	44	46	2	NS
phenylalanine[2]	53[a]	50[b]	27[bc]	16[c]	13[c]	32[bc]	27[bc]	3	0.008
taurine	158	145	120	118	125	120	134	7	NS
tyrosine[2]	87[a]	69[ab]	43[ab]	26[b]	25[b]	33[ab]	48[ab]	5	0.010

[1]Values represent the means of the number of pigs indicated at the top of each column
[2]Overall ANOVA, F-test, $P<0.01$. Values with different superscript letters indicate a significant difference between diet levels (Student Newman Keul's multiple comparisons).
[3] Non-significant is abbreviated to NS.

oxidation. The mean enteral requirement was 0.42 g kg^{-1} d^{-1} when based on phenylalanine as a percentage of the dose oxidized, and 0.44 g kg^{-1} d^{-1} when based on phenylalanine oxidation. The upper 95% confidence interval was estimated and is assumed to meet the methionine requirement of 95% of the population. This safe level of intake in parenteral feeding of methionine was 0.42 g kg^{-1} d^{-1} and enteral feeding, it was 0.48 g kg^{-1} d^{-1}.

There have been numerous studies that have measured methionine requirements in pigs fed a grain-based diet, most commonly a corn-soybean diet. However, it should be noted that little data presently exists on pigs less than 5 kg (NRC, 1998); in fact, the amino acid requirements for these pigs is generally extrapolated from studies done on larger pigs. The present estimate of 0.42 g kg^{-1} d^{-1} is 84% of the NRC (1998) recommendation of 0.50 g kg^{-1} d^{-1}. However, the NRC (1998) recommendation assumes a grain based diet where the amino acids are less available than in the synthetic diet used in the present experiment. Kim and colleagues (1983a) used the indicator amino acid oxidation method with an oral isotope to measure the methionine and the total sulfur amino acid requirement in piglets of approximately the same age (10-14 days old) and weight (3 kg) consuming a diet based on a mixture of amino acids and dried skim milk. These researchers determined both the methionine requirement, when excess cysteine and choline was provided, and the total sulfur amino acid requirement when only methionine was provided, using a broken line regression model. This resulted in a methionine and total sulfur amino acid requirement of 2.7 and 4.4 g/kg diet, respectively. This yields a requirement of 0.26 g kg^{-1} d^{-1} for methionine and 0.43 g kg^{-1} d^{-1} for the total sulfur amino acids. This closely compares to our estimate of 0.42 g kg^{-1} d^{-1} for total

sulfur amino acid, suggesting that methionine, can meet the entire total sulfur amino acid requirement. The total sulfur amino acid requirement has been estimated to be 0.41-0.47 g kg^{-1} d^{-1} (Kroening et al, 1965), 0.51 g kg^{-1} d^{-1} (Braude et al, 1977), and 0.41 g kg^{-1} d^{-1} (Leibholz et al, 1984) in piglets receiving a semipurified (Kroening et al, 1965 and Leibholz et al, 1984) or skim milk-soybean oil diet, respectively (Braude et al, 1977). These estimates were performed on piglets weighing between 1 and 5 kg, and used weight gain, feed efficiency and nitrogen balance to calculate the methionine and total sulfur amino acid requirement. The agreement between these results and the present estimate suggests that there is not a separate requirement for cysteine in piglets of this age.

Statistical comparison of the requirements for methionine by the two different routes of feeding demonstrated that these are significantly different; suggesting that the gut is utilizing approximately 30% methionine and/or possibly cysteine. This is intriguing considering that it has been reported that the metabolism of the sulfur amino acids is accomplished, in its entirety, by only the liver and kidney (Finkelstein, 1990, Stipanuk, 1986). Methionine has not been considered to be catabolized to a large extent by intestinal mucosa because of the low activity of S-adenosyl-L-methionine synthase (Finkelstein, 1990) and the transsulfuration enzyme, cystathionase (Finkelstein, 2000) when their activity is compared with that of the liver. In addition, methionine adenosyltransferase (MAT) has at least three isozymic forms, of which the "high K_m" form (MAT-III) exists only in the liver, suggesting that only the liver (and not the gut) may be unique in its ability to adapt immediately to high levels of methionine

(Finkelstein, 1990). However, these previously reported enzymatic data require reexamination (Wu, 1998).

Previous research has measured a release of methionine (108% of dietary intake) and cysteine (109% of dietary intake) using arterial-venous differences (Garcia and Stipanuk, 1992). This suggests that the gut utilizes negligible amounts of the sulfur amino acids and that the splanchnic release is consistent with the dietary concentration of these amino acids. In addition, these researchers examined arterial-venous differences in the adult rat receiving a purified diet. The apparent negligible release of these amino acids from the gastrointestinal tract may be a result of recycling of mucins and glutathione (cysteine). Within the same study these researchers found a very large uptake of sulfate by the gut; this may indicate that methionine and cysteine are catabolized by the gut to sulfate for conjugation of potentially toxic compounds or for synthesis of sulfated compounds (Krijgsheld et al, 1979).

Stoll and colleagues (1998) reported results that are consistent with the present data. Using the mass balance technique to measure appearance of amino acids in the portal blood in 28 day old piglets administered sow's milk replacer, these researchers reported a 48% appearance of methionine in the portal blood, suggesting a splanchnic methionine uptake of 52% of. In the study by Garcia and Stipanuk (1992), the rats were fed a casein based semi-purified diet containing 8 g/kg methionine, 0.6 g/kg cysteine and 1 g/kg sulfate. In contrast, Stoll et al (1998) fed a milk-based diet and did not report any addition of sulfate. Perhaps, in the absence of sulfate, the sulfur amino acids are catabolized to produce adequate amounts of sulfate for its various functions in the

splanchnic organs. However, both our data and that of Stoll et al (1998) support the idea that there is selective utilization of the sulfur amino acids by the splanchnic tissues.

The differences between previous estimates of amino acid uptake by the gut and our estimate may be due to the differences in dietary components and the age of the piglets. Semi-synthetic and milk replacer diets present both free amino acids and peptides to the enterocyte. In addition, suckling milk fed piglets will also receive immune factors, growth factors, cysteine and glutamine in their diet, all of which may alter intestinal metabolism or affect the estimate of the requirement. The presence of peptides in the enterocyte stimulates transport of individual amino acids (Wenzel et al, 2001) and this may account for a larger amount of amino acids passing into the systemic circulation. In addition, previous data demonstrated that the efficiency of methionine utilization was much lower when an enzymatic hydrosylate of milk vs. an elemental diet was fed enterally when measured using arterial-venous balance (Rerat et al, 1988). Chung and Baker (1992) determined a 1:2, TSAA: lysine ratio for young, 5-10 kg pigs which increases to 1:1.7 in 10-20 kg pigs. Our estimate as a ratio to the lysine requirement as determined by Kim et al (1983b) is 1:2.7, demonstrating that as the pigs' age, the requirement of the total sulfur amino acids increases in proportion to lysine. Thus, differences in diet and age can affect estimates of the requirement and may alter the extent of amino acid utilization by the gut.

The differences seen in methionine requirement between the enterally and parenterally fed piglets may be attributed in part to a decreased use of the intravenously infused amino acids for maintenance of intestinal mass. Parenteral feeding causes gut atrophy (Bertolo et al, 1999, Adeola et al, 1995 and Goldstein et al, 1985), decreased

lower jejunum weight and a reduced fractional and absolute protein synthetic rate compared with enterally fed animals (Dudley et al, 1998). However, in the study by Stoll et al (1998) in which they observed a 52% uptake of methionine, there was no gut atrophy. Furthermore, they estimated less than 20% of amino acids utilization could be accounted for by intestinal protein synthesis. However, differences in the parenteral and enteral tryptophan requirement were not significant (Cvitkovic et al, *unpublished data*) whereas the parenteral threonine requirement (Bertolo et al, 1998) was approximately 45% of the mean enteral requirement and the parenteral branched chain amino acid requirement was approximately 56% of the mean requirement in enterally fed piglets (Elango et al, 2002). The differences in these results are most likely due to the additional metabolic roles of these amino acids in the gut.

There are several potential uses for the sulfur amino acids by the gastrointestinal tissue. Reduced intestinal mass and function resulting from intravenous feeding may account for a large proportion of the observed difference in dietary methionine requirement. A net appearance of taurine in portal blood (Rerat et al, 1988), possibly from its synthesis from cysteine, could account for a proportion of the net utilization of the sulfur amino acids by the splanchnic tissue. In addition, there is glutathione synthesis in the intestinal tissue (Iantomasi et al, 1997) and this may account for a proportion of the sulfur amino acids utilization (Elwyn et al, 1968). Finally, methionine is a major methyl donor *in vivo* and may be used substantially during gut protein synthesis (Finkelstein, 1990) resulting in the end product of the demethylation of methionine, homocysteine, being exported from the splanchnic bed and being catabolized through the transsulfuration pathway by the liver.

In conclusion, a mean methionine requirement when no cysteine was provided was determined to be 0.42 and 0.29 g kg^{-1} d^{-1} for enterally and parenterally fed piglets, respectively, using the indicator amino acid oxidation technique. This estimate of the total sulfur amino acid requirement is 84% of the current recommended intake of 0.5 g kg^{-1} d^{-1} (NRC, 1998) and agrees with previously published data (Kim and Bayley, 1983a, Kroening et al, 1965, and Leibholz, 1984). The differences in parenteral and enteral requirement suggest a 30% utilization of methionine, cysteine, glutathione or sulfate by the gut, which is consistent with some previous estimates of splanchnic use of the sulfur amino acids.

3.1.5 **Literature Cited**

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3.2 DIETARY CYSTEINE REDUCES THE METHIONINE REQUIREMENT BY AN EQUAL PROPORTION IN BOTH PARENTERALLY AND ENTERALLY FED PIGLETS.

3.2.1 Introduction

Whether cysteine is a conditionally indispensable amino acid in either neonates or those receiving total parenteral nutrition remains controversial. In adults, adequate cysteine is synthesized from methionine via the transsulfuration pathway (Finkelstein, 1990). Cysteine is therefore considered to be dispensable in the adult. However, *de novo* cysteine synthesis may be limited in neonatal animals due to the slow maturation of the enzyme cystathionase (EC 4.4.1.1) (Pascal et al, 1972, Sturman et al, 1970, Gaull et al, 1972). Moreover, cysteine synthesis may be further limited in the parenterally fed neonates (Miller et al, 1995) and adults (Stegink and Den Besten, 1972); however, the mechanism responsible for this is unknown. Generally, cysteine is thought to be semi-essential during these periods because of the low plasma cysteine that is associated with TPN feeding, but few studies have evaluated enzyme activity or noted difficulties in maintaining growth. We previously reported the requirement for methionine, in the absence of dietary cysteine, to be 30% higher in enterally compared to parenterally fed neonatal piglets (Shoveller et al, 2003a; Chapter 3.1). This finding suggests that the small intestine or liver utilizes approximately one third of the dietary methionine. In that study, total sulfur amino acids were supplied by methionine alone. The extent to which dietary cysteine can spare the requirement for methionine in neonates must be defined in order to recommend the optimal concentration of cysteine in diets for young pigs and in

pediatric parenteral solutions. In addition, the use of cysteine to meet part of the total sulfur amino acid requirement during TPN would avoid the toxic effects of excess dietary methionine (Moss et al, 1999). Information obtained from neonatal piglets will provide the groundwork for future clinical metabolic studies investigating the optimum intake and ratio of methionine to cysteine in human neonates.

Numerous studies in orally fed animals showed that dietary cysteine can replace part of the methionine requirement. Estimates of cysteine sparing range from 40-70% in growing pigs (Shelton et al, 1951, Curtin et al, 1952, Becker et al, 1955, Mitchell et al, 1968, Baker et al, 1969, Roth et al, 1989, Kim et al, 1983), 50-55% in chicks (Graber et al, 1971a, Graber et al, 1971b, Sasse et al, 1974, Soares et al, 1974), 17-90% in humans (Reynolds et al, 1958, Womack and Rose, 1941, Rose and Wixom, 1955, Clark et al, 1970, DiBuono et al, 2001a) and 50-65% in rats (Rose and Rice, 1939, Rama Rao et al, 1961, Sowers et al, 1972, Shannon et al, 1972). Other data have demonstrated that increases in dietary cysteine down-regulate or inhibit cystathionine β synthase (CBS; EC 4.2.1.22), thereby diminishing transsulfuration and increased methionine retention (Shannon et al, 1972, Finkelstein and Mudd, 1967, Finkelstein et al, 1986, Stipanuk and Benevenga, 1977). A set of studies (Storch et al, 1990, Hiramastu et al, 1994, Raguso et al, 1997, Fukagawa et al, 1998, Raguso et al, 2000) used methionine and cysteine tracers to investigate sulfur amino acid metabolism in humans and all determined that cysteine had no sparing effect on the requirement for methionine. In each of these human studies, methionine and cysteine were provided at the "requirement level" as recommended by the FAO/WHO/UNO (1985). However, recent studies from our group have shown this level of sulfur amino acid intake to be deficient for humans (DiBuono et al, 2001a,

DiBuono et al, 2001b). The lack of cysteine sparing observed in the earlier human studies may have been due to an inadequate supply of dietary methionine, because cysteine can spare methionine only after the obligatory methionine requirement is met. Using double-labeled methionine, DiBuono et al (2003) showed that when a higher methionine intake was provided, dietary cysteine reduced conversion of methionine to cysteine, thereby sparing the dietary requirement for methionine. This research (DiBuono et al, 2001a, 2001b, 2003), demonstrating the methionine sparing capacity of cysteine during oral feeding, now brings the human research into agreement with all of the animal research. However, to the authors' knowledge cysteine sparing has not been previously estimated during parenteral feeding.

We successfully applied the indicator amino acid oxidation technique to measure the amino acid requirements in piglets fed enterally and parenterally (Bertolo et al, 1998, Elango et al, 2002, Brunton et al, 2000). Subsequent studies in human neonates confirmed that findings in the piglet model are valid in parenterally fed human infants (Roberts et al, 2001). In the present study, the objective was to determine the mean methionine requirement when excess dietary cysteine was provided, in neonatal piglets receiving parenteral (TPN) or enteral nutrition by employing the indicator amino acid oxidation technique. Subsequent comparison of these requirements to our previous data (Shoveller et al, 2003; Chapter 3.1) where no dietary cysteine was provided would enable calculation of the cysteine sparing capacity. We hypothesized that the capacity of cysteine to spare the methionine requirement would be approximately 50% in both routes of feeding.

3.2.2 **Materials and Methods.**

3.2.2.1 ***Piglets and study protocol.***

The Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee at the University of Alberta approved all procedures in this study. A total of 28 male Landrace/Large White piglets (Genex Swine Group) were obtained from the University of Alberta, Swine Unit (Edmonton, AB, CAN). Piglets were transported to the Metabolic Unit at the University of Alberta. The piglets were weighed and then anaesthetized with acepromazine (0.5 mg/kg; Atravet™; Ayerst Laboratories, Montreal, PQ) and ketamine hydrochloride (22 mg/kg; Rogarsetic™: Rogar STB Inc., Montreal, PQ) and maintained during surgery with 1% halothane. All piglets were then fitted with two venous catheters (Ed-Art, Don Mills, Canada) using the modified methods of Wykes et al. (1993) and in enterally fed piglets (n=14), gastric catheters were inserted using the method of Rombeau et al. (1984). In all pigs, an infusion catheter was inserted into the left jugular vein and advanced to the superior vena cava just cranial to the heart and a sampling catheter was inserted into the left femoral vein and advanced to the inferior vena cava just caudal to the heart. After surgery, incision sites were treated with a topical antibiotic (Hibitane Veterinary Ointment: Ayerst Laboratories, Montreal, PQ) and an analgesic (0.1 mg/kg Buprenex, Buprenorphrine HCl, Reckitt and Colman Pharmaceutical Inc., Richmond, VA) was given intramuscularly immediately and again 8h-post surgery. Piglets were then put into cotton jackets, which secured the tether to the piglets. The tether is part of the swivel-tether system (Alice King Chatham Medical Arts, Los Angeles, California), that enables the pig to move freely while receiving a

continuous dietary infusion, ensuring that the catheters to do not become tangled or occluded.

3.2.2.2 ***Animal housing.***

Piglets were housed in individual circular cages, 75 cm in diameter, and toys were added to enhance their environment. The animal rooms were maintained at an ambient temperature of 21°C-27°C, with supplemental heat supplied by from heat lamps. The lighting schedule was 12 h of light: 12 h of dark.

3.2.2.3 ***Diet regimen.***

Elemental diets were provided as continuous infusions by pressure sensitive infusion pumps. Piglets received 15 g amino acids $kg^{-1} \cdot d^{-1}$ and 1.1 MJ metabolizable energy $kg^{-1} \cdot d^{-1}$ with glucose and lipid (Intralipid 20%, Fresenius-Kabi, Stockholm, Sweden) each supplying 50% of nonprotein energy intake. The base amino acid profile of the complete elemental diet fed during adaptation (d0 until d5) has been previously described (Shoveller et al, 2003; Chapter 3.1). The amino acid profile was based on human milk protein (Vaminolact: Fresenius-Kabi, Stockholm, Sweden) except phenylalanine and tyrosine which were provided at their estimated safe levels of intake (House et al, 1997a, 1997b) and arginine was provided at 1.2 $g \cdot kg^{-1} \cdot d^{-1}$ (Brunton et al, 1999). The majority (73%) of tyrosine was provided as the dipeptide glycyl-tyrosine (House et al, 1997b). Diet infusion rates were adjusted daily after weighing the piglets. Vitamins were supplied in a commercial solution, MVI Paediatric (Rhone-Poulenc Rorer Canada Inc, Montreal, PQ) which was added to the diet immediately prior to feeding. The cofactors involved in the transsulfuration pathway, vitamin B-12, choline, B-6 and folate were in the MVI solution at approximately 115% of requirement (NRC, 1998).

Piglets also received a mineral solution including zinc, copper, manganese, chromium, selenium and iodide at 200% of the NRC (NRC, 1998) recommendation for piglets.

TPN was initiated immediately following surgery, and increased to full infusion rates (13.5 ml· kg^{-1} ·h^{-1}) by the end of d1 (Shoveller et al, 2003; Chapter 3.1). Complete TPN was continued until 1800 on d5. Piglets were then randomly allocated to one of the test levels of methionine (Parenterally fed: 0.025, 0.05, 0.1, 0.15, 0.2, 0.3, 0.5 or 0.8 g·kg^{-1}· d^{-1}; Enterally fed: 0.025, 0.05, 0.15, 0.25, 0.35, 0.45 or 0.6 g·kg^{-1}· d^{-1}). These dietary methionine concentrations represent a range of 1.65 to 55 mg/g and 1.65 to 40 mg/g of total amino acids for parenterally and enterally fed piglets, respectively. Cysteine was provided as L-cysteine (99% by analysis) in all test diets, providing 0.55 g·kg^{-1}· d^{-1}. All test diet solutions were made isonitrogenous by dissolving the appropriate quantity of L-methionine (at test level) and L-alanine (to maintain isonitrogenous solutions) in 75-mL sterile water; these solutions were then transferred into infusion bags containing 675 mL of concentrated TPN solution without any L-methionine or L-alanine. The solutions were filtered with a 0.22-μm filter (Millipore, Milford, MA). Due to the highly unstable nature of L-cysteine in aqueous solutions, test diets were made immediately prior to infusion of the diet. Piglets were maintained on test diet from 1800h on d 5 until the completion of the oxidation study on d 6. Subsequently, piglets were returned to the complete diet for 24 h. At 1800h on d 7, piglets were randomly assigned to a second test diet and a second oxidation study was performed on d 8. Previously, we determined that a second oxidation indicator amino acid oxidation study 48 h after the first study produces data not different from the first study; therefore, two studies can be conducted

to reduce the number of animals required for an entire indicator amino acid oxidation requirement study (Brunton and Ball, *unpublished data*).

3.2.2.4 ***Tracer infusion, $^{14}CO_2$ collection and analytical procedures.***

Phenylalanine oxidation and flux were determined by a primed (186 kBq (5 μCi/kg)), constant intravenous infusion (130 kBq (3.5 $\mu Ci \cdot kg^{-1} \cdot h^{-1}$)) of a tracer solution containing 92.8 MBq (2.5 mCi)/L of L-[1-^{14}C]phenylalanine (200 MBq (54 mCi/mmol; American Radiolabeled Chemicals, Inc. St. Louis, MO). The constant infusion was 4h, in order to achieve plateau in both blood and breath labeling. Details of infusion protocol, $^{14}CO_2$ collection and blood collection procedures have been described previously (House et al, 1997a). Following the infusion on d8, piglets were killed by injection of 1000 mg of sodium pentobarbital into a venous catheter.

Reverse-phase high performance liquid chromatography with the use of phenylisothiocyanate derivatives was used to measure plasma amino acids. Collection and liquid scintillation counting of radioactive fractions to determine SRA of plasma phenylalanine and tyrosine were completed as previously described (House et al, 1997a). The calculations for intake, oxidation, flux, non-oxidative disposal, release from protein breakdown, balance and percent dose oxidized were as reported previously (House et al, 1997a). SRA for both plasma phenylalanine and tyrosine during the indicator amino acid oxidation study were plotted and plateau values were calculated as the mean SRA at plateau. Plasma concentrations are represented as the mean concentration at each test methionine level.

3.2.2.5 ***Determination of plasma total cysteine (free + bound).***

Plasma total cysteine (protein bound and free) was analyzed according to the reverse phase-HPLC method of Araki and Sako (1987) with modifications as suggested by Gilfix et al. (1997). The inter- and intra-assay coefficients of variation were < 2%.

3.2.2.6 ***Statistical analyses.***

A completely randomized design, with methionine intake serving as the main treatment effect was used in this experiment. The effects of day of indicator amino acid oxidation study (d6 or d8) and weight at study were determined not to be significant ($P>0.10$) using an ANOVA (SAS/STAT, version 8.1, SAS Institute, Cary, NC). If P values were < 0.05 for the F-value of the ANOVA model, significant differences between treatments were determined using Student Newman Keul's multiple comparison procedure. Determination of methionine requirement was performed using a two-way linear crossover model, as described previously (Seber, 1977, Ball and Bayley, 1984). Regression analysis variables were dietary concentration of methionine as the independent variable and percentage of dose oxidized and phenylalanine oxidation as the dependent variables. The 95% confidence intervals, for the estimation of a safe level of intake, were also determined.

To compare the breakpoints, or requirement estimates, between parenterally and enterally fed piglets, we treated the breakpoint as a sample mean and used the pooled two-sample t procedure (Pagano and Gavreau, 2000). Subsequently, we statistically compared the present results with those from a previous study (Shoveller et al, 2003; Chapter 3.1) to calculate the sparing capacity of cysteine within route of feeding (**Table 3.2.1**). On the basis of the assumption that the subjects were derived from the same population and identical procedures were used, the true variance was assumed to be the.

Table 3.2.1: Comparison of the methionine requirements (g-kg^{-1}-d^{-1}) and % of dose oxidized at plateau determined by indicator amino acid oxidation in neonatal piglets receiving an elemental diet by either enteral or parenteral feeding routes and no dietary cysteine or excess cysteine.

Route of feeding	MET without CYS[1]	MET with (0.55 g·kg^{-1}· d^{-1}) CYS	Sparing capacity of CYS
Enterally fed			
Requirement[2]	0.42 ± 0.033*§	0.25 ± 0.055*	40%
Plateau % of dose oxidized[3]	0.44 ± 0.10	0.65 ± 0.34	
Parenterally fed			
Requirement	0.29 ± 0.067§	0.18 ± 0.048	38%
Plateau % of dose oxidized	0.44 ± 0.33	0.69 ± 0.39	
Gut utilization	31%	28%	

[1] Data from Shoveller et al, 2003 (2003).
[2] Values represent the mean requirement ± 1 SD that was calculated using a two-way linear crossover model.
[3] Values represent the mean % of dose oxidized at plateau (after the breakpoint) ± 1 SD that was calculated using an ANOVA. There were no differences among dietary treatments.
* Overall two sample t procedure, $P<0.01$. Values with a * indicate a significant difference between mean requirements within the column (enterally fed vs. parenterally fed).
§ Overall two sample t procedure, $P<0.01$. Values with a § indicate a significant difference between mean requirements within the row (MET without CYS vs. MET with CYS).

same. Calculation of pooled variance was used to average each sample variance with weights equal to its degrees of freedom. Therefore, pooled variance for the two groups was determined and used to test if the two breakpoints were different using a pooled two-sample t-procedure.

3.2.3 **Results**

All piglets were healthy and active during the course of the study. Piglet age and weight upon arrival (2 d, 1.63 kg, pooled SE= 0.02) and at study (9 d, 2.61 kg, pooled SE= 0.035) were not significantly different (P>0.05) among diet levels or between routes of feeding. Average daily gain for the 5-day adaptation period prior to test diet initiation was not significantly different between routes of feeding (144 ± 5.77 g/d (SE) for parenterally and 161 ± 3.25 g/d (SE) for enterally fed piglets).

3.2.3.1 ***Parenteral and enteral methionine requirement.***

Breath $^{14}CO_2$, plasma phenylalanine SRA and plasma tyrosine SRA reached plateau 2h after the initiation of the primed, constant infusion in all pigs. The ratio of plasma tyrosine SRA/ plasma phenylalanine SRA was not significantly different among diet treatments; thus, the rate of conversion of phenylalanine to tyrosine did not affect the breakpoint estimate.

3.2.3.2 ***Parenteral methionine requirement.***

Phenylalanine flux, intake, non-oxidative disposal and release from protein were not significantly different (P>0.05) among dietary treatments (**Table 3.2.2**). The similarity in flux among dietary treatments indicates that the differences observed in

Table3.2.2: *Parenterally fed treatment*: phenylalanine kinetics of piglets receiving total parenteral nutrition with graded levels of dietary methionine and excess cysteine[1]

	Methionine Intake ($g \cdot kg^{-1} \cdot d^{-1}$)									
	0.025	0.05	0.1	0.15	0.2	0.3	0.5	0.8	pooled SE	ANOVA P value
n	3	4	4	4	4	4	2	3		
($\mu mol \cdot kg^{-1} \cdot h^{-1}$)										
Flux	181	254	233	218	151	284	231	289	19.3	NS[2]
Intake	123	108	122	113	112	115	111	119	1.8	NS
Oxidation	15	9	8	5	1	2	3	2	1.4	0.11
Non-Oxidative Disposal	166	245	224	213	150	282	228	288	19.1	NS
Release from protein	59	146	111	105	75	168	120	171	18.9	NS

[1]Values represent the means of the number of pigs indicated at the top of each column.
[2] Non-significant is abbreviated to NS ($P>0.05$).

phenylalanine oxidation reflect a shift in partitioning between oxidation and protein synthesis. Furthermore, the lack of difference in Tyrosine: Phenylalanine SRA indicates that when dietary tyrosine was in excess, phenylalanine was partitioned between non-oxidative disposal and oxidation in proportion to the changes in protein synthesis (House et al, 1997a, Shinman and Gray, 1998). Phenylalanine oxidation, expressed as percent of the dose oxidized was affected by methionine intake (P= 0.03, **Table 3.2.3**) and was approaching significance (P=0.06) when expressed as absolute oxidation (Table 3.2.2). As methionine intake increased from 0.05 $g \cdot kg^{-1} \cdot d^{-1}$ to 0.18 $g \cdot kg^{-1} \cdot d^{-1}$, phenylalanine oxidation (% of dose) decreased (P<0.05). Further increases in methionine intake did not affect phenylalanine oxidation (P>0.05, slope was not different from zero, **Figure 3.2.1**).

To determine the methionine requirements, the data points were partitioned between two distinct regression lines (Figure 3.2.1). The final regression was chosen as the model that produced the highest regression coefficients for the dependent variables. The breakpoint is the point at which the two regression lines intersected and gives an estimate of the mean methionine requirement. The variation associated with the regression lines is used to calculate the corresponding confidence intervals. The breakpoint estimate for phenylalanine oxidation rate (requirement = 0.17 $g \cdot kg^{-1} \cdot d^{-1}$, 95% CI: 0.08-0. $g \cdot kg^{-1} \cdot d^{-1}$) was similar to that for phenylalanine oxidation rate as a percentage of dose (Figure 3.2.1; (requirement = 0.18 $g \cdot kg^{-1} \cdot d^{-1}$, 95% CI: 0.08-0.27 $g \cdot kg^{-1} d^{-1}$).

Methionine intake did not influence the plasma free amino acid concentrations of methionine and cysteine (P>0.05, **Table 3.2.4** and **Figure 3.2.2**), similar to a previous study where we altered dietary methionine (Shoveller et al, 2003;Chapter 3.1). Alanine was used to make the diets isonitrogenous and therefore the intake of alanine and plasma

Table 3.2.3: ***Parenterally fed treatment:*** **plateau values for $^{14}CO_2$ recovery and phenylalanine (Phe) and tyrosine (Tyr) specifi radioactivity (SRA) in plasma of piglets receiving total parenteral nutrition with graded levels of dietary methionine and excess cysteine[1]**

	Methionine Intake ($g \cdot kg^{-1} \cdot d^{-1}$)									
	0.025	0.05	0.1	0.15	0.2	0.3	0.5	0.8	pooled	ANOV
n	3	4	4	4	4	4	2	3	SE	P valu
percent of phenylalanine oxidized[2]	7.07[a]	3.48 [ab]	3.73 [ab]	2.47 [ab]	0.98[b]	0.65[b]	1.15[b]	0.58[b]	0.55	0.
Expired $^{14}CO_2$, (volume corrected)[4], $kBq \cdot kg^{-1} \cdot h^{-1}$	6.72	4.08	4.91	2.90	1.25	0.85	1.52	0.74	0.57	0.0
Phe SRA, (MBq/mol)	680	530	591	593	645	425	475	441	158	NS[3]
Tyr SRA (MBq/mol)	100	48	67	43	32	37	13	60	6.7	NS
Tyr SRA: Phe SRA	20.3	13.1	10.6	7.0	4.4	10.4	2.7	7.0	1.6	NS

[1] Values represent the means of the number of pigs indicated at the top of each column.
[2]Overall ANOVA, F-test, $P<0.01$. Values with different superscript letters indicate a significant difference among diet levels (Student Newman Keul's' multiple comparisons).
[3] Non-significant is abbreviated to NS ($P>0.05$).
[4] Values corrected for a 93.3 % bicarbonate retention factor.

Figure 3.2.1: ***Parenteral methionine requirement when excess dietary cysteine was supplied:*** **oxidation of L-[1-^{14}C]phenylalanine as a percentage of dose in parenterally fed piglets receiving graded levels of methionine and excess cysteine (n=32). The break-point value was 0.18 g-kg^{-1}-d^{-1}.**

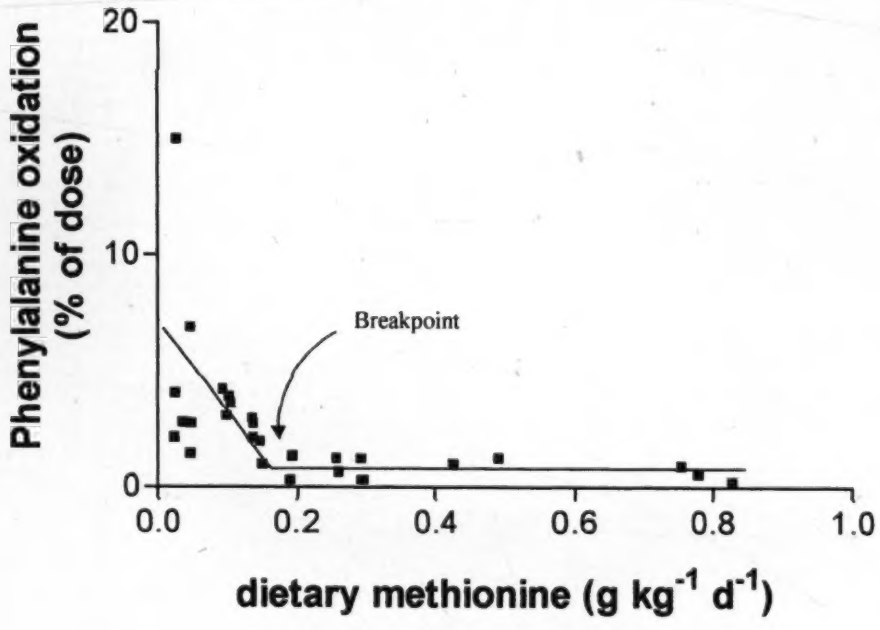


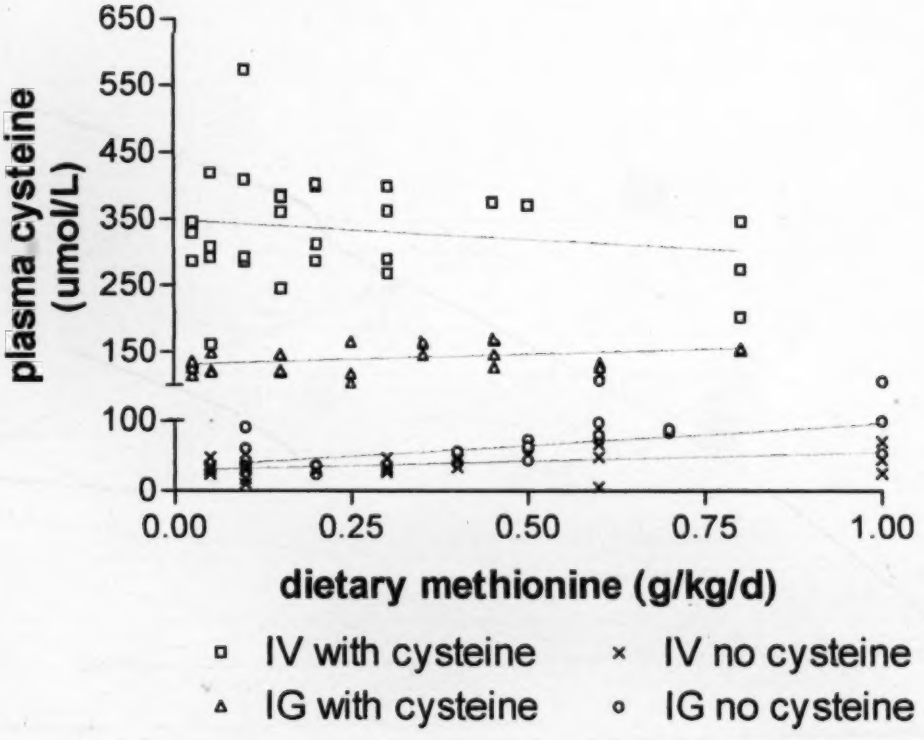
Table 3.2.4 : Parenterally fed treatment: plasma amino acid concentrations (µmol/L) of piglets receiving total parenteral nutrition with graded levels of dietary methionine and excess cysteine1

	Methionine Intake ($g \cdot kg^{-1} \cdot d^{-1}$)									
amino acid	0.025	0.05	0.1	0.15	0.2	0.3	0.5	0.8	pooled	ANOVA
n	3	4	4	4	4	4	2	3	SE	P value
(µmol/L)										
methionine	41	44	44	47	39	36	39	32	2	NS
taurine	913^{a}	746^{a}	460^{b}	365^{b}	253^{b}	249^{b}	295^{b}	347^{b}	47	<0.0001
serine	1245^{a}	1418^{a}	1042^{a}	1031^{a}	635^{b}	633^{b}	539^{b}	794^{b}	81	0.03
phenylalanine	257^{a}	233^{a}	174^{b}	170^{b}	121^{bc}	78^{c}	90^{c}	84^{c}	13	<0.0001
tyrosine	612^{a}	363^{a}	247^{b}	127^{b}	98^{b}	51^{b}	83^{b}	54^{b}	40	0.0002
lysine	1469	861	1256	1029	913	796	829	898	77	NS
leucine	497^{a}	411^{ab}	380^{ab}	305^{bc}	317^{bc}	253^{bc}	268^{bc}	208^{c}	20	0.0005
isoleucine	293^{a}	243^{ab}	232^{ab}	138^{c}	170^{bc}	130^{c}	114^{c}	124^{c}	13	0.0002
valine	695^{a}	560^{b}	452^{c}	365^{cd}	330de	256de	277de	221^{e}	30	<0.0001
threonine	923	888	830	919	766	630	730	605	46	NS
histidine	259^{a}	246^{a}	163^{b}	125^{b}	78^{b}	71^{b}	86^{b}	65^{b}	16	<0.0001
tryptophan	91	50	40	41	51	48	48	46	5	NS

arginine	314^{a}	260^{ab}	255^{ab}	142^{b}	177^{ab}	179^{ab}	243^{ab}	181^{ab}	14	0.01
proline	2768^{a}	2370^{a}	1634^{b}	1314^{b}	960^{b}	941^{b}	950^{b}	1309^{b}	137	<0.0001
citrulline	333^{a}	261^{b}	176^{c}	129^{cd}	97^{d}	92^{d}	95^{d}	98^{d}	17	<0.0001
ornithine	165	156	226	214	163	121	135	133	13	NS
glutamate	1250^{a}	1104^{ab}	1240^{a}	708^{ab}	298^{b}	362^{ab}	388^{ab}	402^{ab}	94	0.002
glutamine	870^{a}	785^{a}	509^{ab}	315^{b}	132^{b}	83^{b}	61^{b}	86^{b}	67	<0.0001
alanine	1169^{ab}	1056^{ab}	888^{ab}	910^{ab}	822^{ab}	696^{b}	1451^{a}	895^{ab}	55	0.05
asparagine	100^{a}	80^{ab}	51^{ab}	39^{b}	22^{b}	30^{b}	18^{b}	19^{b}	7	0.004
aspartate	135	266	153	60	30	42	41	37	27	NS
glycine	4616	3330	2398	2329	2029	2253	2423	1514	282	NS

[1] Values represent the means of the number of pigs indicated at the top of each column.
[2]Overall ANOVA, F-test, P<0.01. Values with different superscript letters indicate a significant difference between diet levels (Student Newman Keul's multiple comparisons).
[3] Non-significant is abbreviated to NS (P>0.05).

Figure 3.2.2: The correlation between plasma total cysteine (free + bound) and dietary methionine in intragastrically fed neonatal piglets receiving either no dietary cysteine or excess dietary cysteine, or intravenously fed piglets receiving either no dietary cysteine or excess dietary cysteine. Plasma cysteine was highest in IV plus cysteine, intermediate in the IG plus cysteine group, lower in the IG without cysteine and lowest in the IV without cysteine group (P<0.05).



alanine concentrations decreased as dietary methionine increased (Table 3.2.4). The plasma concentrations of tyrosine, taurine, serine, leucine, isoleucine, valine, histidine, arginine, proline, citrulline, glutamate, glutamine and asparagine significantly decreased as dietary concentrations of methionine increased (Table 3.2.4). Plasma concentrations of phenylalanine decreased with diets containing 0.025 to 0.3 g methionine kg^{-1} d^{-1} and then did not change with further increases in methionine intake.

3.2.3.3 ***Enteral methionine requirement.***

Phenylalanine flux, intake, non-oxidative disposal and release from protein were not significantly different ($P>0.05$) among dietary treatments (**Table 3.2.5**). Phenylalanine oxidation, expressed as percent of the dose oxidized was significantly influenced by methionine intake ($P<0.005$, **Table 3.2.6**). As methionine intake increased from 0.1 $g \cdot kg^{-1} \cdot d^{-1}$ to 0.30 $g \cdot kg^{-1} \cdot d^{-1}$, phenylalanine oxidation (% of dose) decreased ($P<0.01$, **Figure 3.2.3**). Further increases in methionine intake did not affect phenylalanine oxidation ($P>0.05$, slope was not different from zero; Figure 3.2.3). The breakpoint estimate for phenylalanine oxidation rate (requirement = 0.21 $g \cdot kg^{-1} \cdot d^{-1}$, 95% CI: 0.02-0.37 $g \cdot kg^{-1} \cdot d^{-1}$) was similar to that for phenylalanine oxidation rate as a percentage of dose (Figure 3.2.3; requirement 0.25 $g \cdot kg^{-1} \cdot d^{-1}$, 95% CI: 0.14-0.36 $g \cdot kg^{-1} \cdot d^{-1}$).

Similar to the parenteral experiment, methionine intake did not influence the plasma free concentrations of methionine ($P>0.05$, **Table 3.2.7**). The plasma concentrations of tyrosine, taurine, serine, isoleucine, valine, histidine, proline, citrulline, glutamate, glutamine, asparagine and aspartate all decreased significantly as dietary concentrations of methionine increased (Table 3.2.7). With methionine intakes of 0.025

Table 3.2.5: ***Enterally fed treatment*****: phenylalanine kinetics (μmol kg^{-1} h^{-1}) of piglets receiving total nutrition intragastrically with graded levels of dietary methionine and excess cysteine[1]**

	Methionine Intake ($g \cdot kg^{-1} \cdot d^{-1}$)								
	0.025	0.05	0.15	0.25	0.35	0.45	0.6	pooled SE	ANOVA P value
n	4	4	4	4	4	4	3		
($\mu mol \cdot kg^{-1} \cdot h^{-1}$)									
Flux	325	235	175	217	236	212	160	40	NS[2]
Intake	109	122	124	115	133	119	127	2.9	NS
Oxidation	8.6	8.5	4.4	1.62	1.1	1.4	1.35	1.1	NS
Non-Oxidative Disposal	316	226	170	216	234	210	158	40	NS
Release from protein	216	113	51	102	101	92	32	40	NS

[1] Values represent the means of the number of pigs indicated at the top of each column.
[2] Non-significant is abbreviated to NS (P>0.05).

Table 3.2.6: ***Enterally fed treatment:*** **plateau values for $^{14}CO_2$ recovery and phenylalanine (Phe) and tyrosine (Tyr) specific radioactivity (SRA) in plasma of piglets receiving total nutrition intragastrically nutrition with graded levels of dietary methionine and excess cysteine[1]**

	Methionine Intake ($g \cdot kg^{-1} \cdot d^{-1}$)								
	0.025	0.05	0.15	0.25	0.35	0.45	0.6	pooled SE	ANOVA P value
n	4	4	4	4	4	4	3		
percent of phenylalanine oxidized[2]	3.94^{ab}	4.82^{a}	2.40^{ab}	0.62^{b}	0.56^{b}	0.61^{b}	0.80^{b}	0.43	0.009
Expired $^{14}CO_2$, (volume corrected), kBq $kg^{-1} \cdot h^{-1}$ [2, 4]	4.55^{ab}	5.67^{a}	2.43^{ab}	0.72^{b}	0.65^{b}	0.72^{b}	0.95^{b}	0.49	0.005
Phe SRA, (MBq/mol)	4055	1318	1908	848	626	833	2603	468	NS[3]
Tyr SRA (MBq/mol)	58	62	32	60	22	38	13	8.3	NS
Tyr SRA: Phe SRA	5.0	8.9	5.8	8.3	2.9	8.7	1.4	1.2	NS

[1] Values represent the means of the number of pigs indicated at the top of each column.
[2]Overall ANOVA, F-test, $P<0.01$. Values with different superscript letters indicate a significant difference among diet levels (Student Newman Keul's multiple comparisons).
[3] Non-significant is abbreviated to NS ($P>0.05$).
[4] Values corrected for a 93.3 % bicarbonate retention factor.

Figure 3.2.3: ***Enteral methionine requirement when excess dietary cysteine was supplied:*** **oxidation of L-[1-^{14}C]phenylalanine as a percentage of dose in enterally fed piglets receiving graded levels of methionine and excess cysteine (n=32). The break-point value was 0.25 g-kg^{-1}-d^{-1}.**

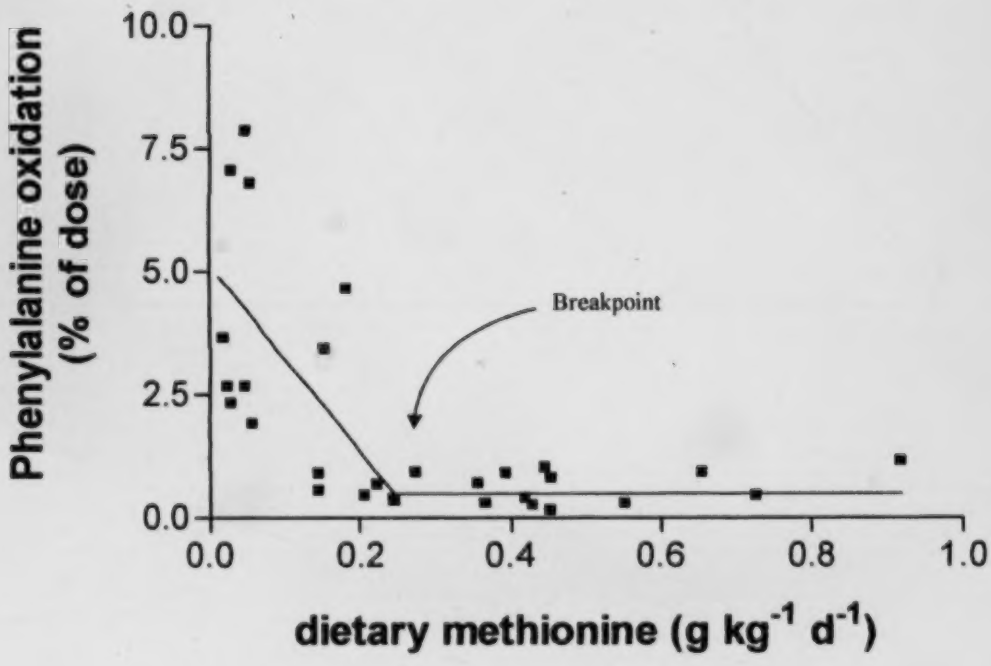


Table 3.2.7: ***Enterally fed treatment*****: plasma amino acid concentrations (μmol/L) of piglets receiving total nutrition intragastrically with graded levels of dietary methionine and excess cysteine[1]**

	Methionine Intake ($g \cdot kg^{-1} \cdot d^{-1}$)								
amino acid	0.025	0.05	0.15	0.25	0.35	0.45	0.6	pooled SE	ANOVA P value
n	4	4	4	4	4	4	3		
(μmol/L)									
methionine	44	51	50	44	47	47	42	1	NS
taurine	653^{a}	633^{a}	333^{b}	255^{b}	319^{b}	364^{b}	350^{b}	31	<0.0001
Serine	717^{ab}	787^{a}	506^{bc}	395^{c}	391^{c}	358^{c}	387^{c}	39	0.002
phenylalanine	203^{a}	156^{ab}	104^{bc}	67^{c}	64^{c}	66^{c}	80^{c}	11	<0.0001
tyrosine	207^{a}	237^{a}	128^{b}	53^{b}	54^{b}	47^{b}	94^{b}	15	<0.0001
Lysine	1082	1134	923	783	950	880	852	44	NS
leucine	583	529	419	409	407	418	376	22	NS
isoleucine	306^{a}	258^{ab}	197^{b}	175^{b}	174^{b}	171^{b}	167^{b}	12	0.002
Valine	703^{a}	663^{a}	419^{b}	361^{b}	356^{b}	352^{b}	374^{b}	31	<0.0001
threonine	1003	1504	1265	1115	1145	1283	1048	56	NS
histidine	131^{a}	135^{a}	86^{b}	73^{b}	88^{b}	81^{b}	76^{b}	6	0.01
tryptophan	74	71	63	62	53	42	65	4	NS

arginine	273	246	213	264	247	195	313	15	NS
proline	1435^{ab}	1490^{a}	1109^{abc}	947^{c}	1082^{abc}	1115^{abc}	982^{bc}	52	0.01
citrulline	202^{a}	187^{ab}	134^{b}	120^{b}	134^{b}	132^{b}	124^{b}	8	0.006
ornithine	268	298	245	204	210	245	238	14	NS
glutamate	623^{b}	1264^{a}	413^{b}	327^{b}	293^{b}	393^{b}	374^{b}	63	<0.0001
glutamine	827^{a}	613^{a}	319^{b}	161^{b}	186^{b}	182^{b}	161^{b}	54	<0.0001
alanine	1035	1062	1209	1050	1218	1223	1201	40	NS
asparagine	84^{a}	78^{a}	35^{b}	23^{b}	22^{b}	25^{b}	21^{b}	5	<0.0001
aspartate	45^{b}	83^{a}	27^{b}	28^{b}	27^{b}	29^{b}	25^{b}	4	<0.0001
glycine	3247	3385	2427	1744	1709	1502	1668	224	NS

[1] Values represent the means of the number of pigs indicated at the top of each column.
[2]Overall ANOVA, F-test, P<0.01. Values with different superscript letters indicate a significant difference between diet levels (Student Newman Keul's multiple comparisons).
[3] Non-significant is abbreviated to NS (P>0.05).

to 0.25 $g \cdot kg^{-1} \cdot d^{-1}$ plasma concentrations of phenylalanine decreased and then did not change with further increases in methionine intake.

3.2.3.4 ***Differences between methionine requirements.***

The comparison between means (breakpoint or requirement) resulted in a significant difference ($P<0.005$). This difference indicates that when excess cysteine is provided, the intravenous methionine requirement is 72% of the enteral requirement. Further comparison to our previous study (Shoveller et al, 2003; Chapter 3.1) in which we measured the methionine requirement without cysteine resulted in significant differences within route of feeding ($P<0.05$, Table 3.2.1). These differences indicate that cysteine can spare the dietary methionine requirement by approximately 40% in both enteral and parenteral feeding.

3.2.3.5 ***Effects of cysteine supplementation on whole body protein synthesis.***

This was determined by comparing the data in the current study with the data from our recently reported work (Shoveller et al, 2003; Chapter 3.1), conducted under identical experimental conditions, in which piglets were fed methionine only. We compared the mean percentage of the phenylalanine dose oxidized at plateau among the four dietary treatments. Plateau oxidation (as a % of dose) was not different among dietary treatments ($P>0.05$); therefore, whole body protein synthesis was not different whether or not dietary cysteine was supplied. We can therefore conclude that dietary cysteine is not a dietary indispensable amino acid in neonatal piglets.

3.2.3.6 ***Plasma total cysteine.***

Dietary methionine intake was positively correlated with total plasma cysteine concentrations in enterally fed piglets (IG-CM) ($P<0.05$; Figure 3.2.2), but was not

correlated in the parenterally fed piglets (IV-CM) ($P>0.05$; Figure 3.2.2). We also measured the total plasma cysteine in piglets that received stepwise methionine intakes during both parenteral and enteral feeding with no dietary cysteine (Shoveller et al, 2003; Chapter 3.1). These new data are included in the present paper for a more complete comparison of the effects of methionine intake and route of feeding on plasma total cysteine concentrations (Figure 3.2.2). When piglets were fed methionine without dietary cysteine (Shoveller et al, 2003; Chapter 3.1), dietary methionine intake was positively correlated to total plasma cysteine during enteral (IG-M) but not parenteral (IV-M) feeding. Plasma cysteine was significantly different among all treatments; highest in the IV-CM group (334 μmol/L), which was greater than the IG-CM (139 μmol/L). Both groups fed excess cysteine had greater plasma cysteine concentrations than the groups fed methionine alone; the IG-M group has higher concentrations (~ 65 μmol/L) than the IV-M group(~ 38 μmol/L) ($P<0.01$).

3.2.4 **Discussion**

The neonatal piglet model, in combination with the indicator amino acid oxidation technique, was successfully applied to determine the parenteral lysine (House et al, 1998) and the parenteral and enteral threonine (Bertolo et al, 1998) and branched chain (Elango et al, 2002) requirements. These reports contribute to our long-term goal of redefining the amino acid requirements of both enterally and parenterally fed neonates using sensitive carbon oxidation techniques (Brunton et al, 2000). Our objective in the present study was to quantify the cysteine sparing capacity in a neonatal model under well-controlled conditions. To do this, we used the indicator amino acid oxidation

technique to measure the methionine requirement when excess cysteine was provided. Subsequent comparison to our previous study in piglets that were fed diets containing only methionine (Shoveller et al, 2003; Chapter 3.1) allowed for the calculation of the maximal sparing capacity of cysteine (Table 3.2.1). This comparison suggests that cysteine can reduce the methionine requirement by 38 and 40% in both parenteral and enteral routes of feeding, respectively (Table 3.2.1). In the present study we varied the dietary intakes of methionine from 0.025-g·kg^{-1} d^{-1} (5% of the total sulfur amino acid requirement) to intakes in excess of the total sulfur amino acid requirement using a diet where all the amino acid concentrations were known. Furthermore, an elemental diet was used, which is likely 100% bioavailable. Therefore, we met all the conditions necessary to accurately calculate the cysteine sparing capacity (Chung and Baker, 1992). The absolute amount of sparing in the parenteral and enteral routes of feeding was 0.11 and 0.17 g methionine kg^{-1} d^{-1}, respectively.

The mean parenteral methionine requirement when excess cysteine was provided, as determined by a two-phase linear crossover model, was 0.18 g·kg^{-1}·d^{-1}. The mean enteral requirement was 0.25 g·kg^{-1}·d^{-1}. The upper 95% confidence interval was estimated and is assumed to meet the methionine requirement of 95% of the population. This safe level of intake of methionine was determined to be 0.27 g·kg^{-1} d^{-1} and 0.36 g·kg^{-1}·d^{-1}, in parenteral and enteral feeding, respectively. The significant difference between breakpoints suggests that the gut is using approximately 28% of the total sulfur amino acid requirement (Table 3.2.1). This closely matches our previous estimate of splanchnic extraction (Shoveller et al, 2003; Chapter 3.1) of 31% of the methionine intake (Table 3.2.1).

When measuring the amount of cysteine needed to optimize performance, one must account for the differences in molecular weight between methionine and cysteine. Because of this molecular weight difference, the efficiency of methionine in meeting the biological need for cysteine on a weight or concentration basis is in the order of 80%, whereas an assumption of 100% is frequently (Chung and Baker, 1992) and incorrectly used. Therefore, our present estimate of 40% of the methionine requirement spared on a weight basis equates to cysteine comprising 50% of the total sulfur amino acid requirement.

In growing pigs, the maximal proportion of the sulfur amino acid requirement that can be met by cysteine has been estimated to range between 40-70% (Shelton et al, 1951, Curtin et al, 1952, Becker et al, 1955, Mitchell et al, 1968, Baker et al, 1969, Roth et al, 1989, Kim et al, 1983). Presently, the National Research Council (1998) suggests that methionine and cysteine be supplied in a 50:50 ratio (weight: weight) for swine of all ages. However, few data exist on pigs less than 3 kg; therefore, the majority of recommendations for these piglets are extrapolated from experiments that determined the requirements of older pigs. Our estimate of the enteral cysteine sparing capacity (approximately 40% in both routes of feeding) is comparable to previous estimates in growing pigs. Kim and colleagues (1983) used the indicator amino acid oxidation method and oral isotope dosing to measure the methionine requirements in piglets of a similar age (10-14 days old) and weight (3 kg) as those in the present study. The piglets were fed a diet based on free amino acids and dried skim milk. The methionine requirement, when excess cysteine and choline were provided, was 2.7 g/kg diet. This calculates into a requirement of 0.26 g· methionine $kg^{-1} \cdot d^{-1}$. Within the same study they

measured the methionine requirement to be 0.43 $g \cdot kg^{-1} d^{-1}$ when no dietary cysteine was provided. This is remarkably similar to our estimates of 0.42 $g \cdot kg^{-1} d^{-1}$ for methionine alone and 0.25 $g \cdot kg^{-1} d^{-1}$ when cysteine is supplied in excess. Chung and Baker (1992) measured the maximal proportion of the dietary sulfur amino acids that could be supplied by cysteine in 10 kg pigs fed a chemically defined amino acid diet. Methionine:cystine ratios of 60:40 and 50:50 (weight:weight) supported greater weight gain than those observed in pigs fed 40:60. The 60:40 and 50:50 ratio is comparable to our estimate, resulting in cysteine meeting 40% of the total sulfur amino acid requirement on a methionine weight basis. Given the agreement between these results, it is apparent that cysteine cannot supply greater than 50% of the total sulfur amino acid requirement. Conversely, cysteine can replace 40% of the methionine requirement, if methionine were meeting 100% of the total sulfur amino acid requirement. Our data demonstrate for the first time that this recommendation also applies to parenteral feeding, for which the estimate as a proportion of the methionine requirement was not different.

The cysteine sparing effect on the methionine requirement that we observed in the present study is supported by *in vitro* data that has demonstrated a correlation between increases in dietary cysteine and the concomitant reduction in the activity of the CBS enzyme in the liver. Supplemental dietary cysteine significantly reduced hepatic CBS activity (Finkelstein and Mudd, 1967, Finkelstein et al, 1986, Stipanuk and Benevenga, 1977). Increasing dietary cysteine up to 50% of the sulfur amino acids decreased CBS activity but did not affect growth (Shannon et al, 1972). This reduction in CBS activity results in a reduction in the rate of transsulfuration and subsequently decreases the endogenous synthesis of cysteine. Therefore, there is a reduction in transsulfuration and

the dietary methionine requirement is reduced when dietary cysteine is increased. In the present study, the similarity in sparing capacities between routes of feeding suggests that the rate of transsulfuration was not different when first pass metabolism by the small intestine is bypassed.

Cysteine has been reported to be conditionally essential for premature neonates due to the low activity of the transsulfuration enzyme cystathionase in fetal tissue and a greater concentration of its precursor cystathionine (Gaull et al, 1972, Sturman et al, 1970, Pohlandt, 1974). In addition, other research has demonstrated that plasma cyst(e)ine concentrations are dramatically decreased in the neonate administered TPN (Zlotkin and Anderson, 1982). However, cystathionase activity is positively correlated with the gestational and postnatal age of the infant (Zlotkin et al, 1981) suggesting that term infants may not have limited cysteine synthesis. In addition, cystathionase activity is present in both the adrenals and kidneys of both premature and term infants. *In vitro* experiments indicate that the premature infant is potentially capable of synthesizing adequate endogenous cysteine if provided with adequate dietary methionine (Zlotkin et al, 1981). Furthermore, Zlotkin et al (1981) reported that cysteine supplementation to TPN did not result in an improvement in nitrogen retention in term or premature infants, but it did normalize plasma concentrations of cysteine. In our studies, there was no evidence to support the claim that cysteine is indispensable in neonates; however, we did observe the lowest plasma cysteine concentrations in TPN fed piglets fed no cysteine (Shoveller et al, 2003; Chapter 3.1; Figure 3.2.2). The oxidation of the indicator amino acid (phenylalanine), at adequate methionine concentrations, was similar in our two studies with and without dietary cysteine (Table 3.2.1). This suggests that there is not a

separate requirement for cysteine. A lower baseline oxidation when cysteine was provided would have indicated an essential requirement for cysteine that could not be met by methionine. However, if cysteine use for protein synthesis has priority over its use for the synthesis of products, such as taurine and glutathione, then we may not have measured the total requirement for methionine for both protein synthesis and conversion to specific products.

Plasma total cysteine concentrations were profoundly different among all treatment groups (Figure 3.2.2). The IG-M treatment had significantly higher plasma cysteine concentrations than the IV-M treatment, indicating that either the gut produces cysteine and/or parenterally fed piglets require more cysteine for glutathione, taurine or sulfate synthesis. A positive correlation between dietary methionine intake and plasma cysteine concentrations in the enterally, but not the parenterally, fed treatments, further suggests that the gut is involved in cysteine synthesis. In addition, we observed the lowest concentrations in piglets administered the IV-M treatment. However, piglets administered the IV-CM treatment had higher plasma cysteine concentrations than those given the IG-CM treatment, suggesting that the gut was responsible for a reduction in the synthesis and release of cysteine into the systemic circulation when cysteine was given enterally. All the enzymes for the transsulfuration pathway are present in the gut (Finkelstein, 1990); therefore, the presence of enterally fed cysteine may spare the catabolism of methionine via the transsulfuration pathway (via negative inhibition of CBS) whereas the cysteine released from the gut incrementally increased with increased concentrations of the dietary sulfur amino acids. The low plasma cysteine concentrations found in the IV-M treatment agree with previous studies that have found low plasma

cysteine concentrations during parenteral feeding in neonates (Miller et al, 1995, Van Goudoever et al, 1995), young rabbits (Moss et al, 1995) and adults (Stegink and Den Besten, 1972). Infants who received TPN supplemented with cysteine had 60% greater plasma cysteine than an unsupplemented group, even though nitrogen balance did not change (Zlotkin et al, 1981). Furthermore, in piglets administered the IG-CM treatment, plasma cysteine concentrations were consistent with dietary concentrations of cysteine, similar to results obtained in rats fed a diet that supplied both methionine and cysteine (Garcia and Stipanuk, 1992). The liver plays the dominant role in cysteine catabolism (Stipanuk et al, 2002), thus it is unlikely that the difference found between IG-CM and IV-CM treatments was due to extensive cysteine oxidation in the gut. However, cysteine is metabolized to some extent by all tissues, including the small intestine, so the gut may have been partly responsible for cysteine catabolism. Clearly, the gut is involved in the metabolic regulation of the sulfur amino acids; and likely synthesizes cysteine when the diet is lacking in cysteine and reduces its synthesis from methionine when the diet contains adequate amounts of cysteine.

In the present study, the concentration of many plasma amino acids decreased as methionine intake increased (Tables 3.2.4 and 3.2.7). We have previously reported a similar response to dietary branched chain amino acids (Elango et al, 2002). This change in plasma amino acids may represent the increasing incorporation of these amino acids into protein with increasing intake of the limiting amino acid. In addition, at low methionine intakes we observed significant increases in the plasma concentrations of glutamate and glutamine, indicating that there was an increased need to carry nitrogen to the urea cycle for disposal. Taurine, a beta amino acid that is not incorporated into

protein, decreased as methionine intake increased in both parenteral and enteral feeding (Tables 3.2.4 and 3.2.7), reached its lowest concentrations at the diet level closest to the estimated breakpoint (0.2 and 0.25 $g \cdot kg^{-1} d^{-1}$, respectively) and then increased at the highest diet levels. The excess cysteine at low and high methionine intakes may have been shunted towards taurine synthesis. This implies that as dietary methionine was increased, the incorporation of cysteine into protein increased; thus, the synthesis of taurine decreased until the concentration of methionine reached requirement and then taurine concentrations rose once again. Indeed, hepatic cysteine dioxygenase activity and urinary excretion of taurine were elevated in rats fed diets with excess cysteine (Daniels and Stipanuk, 1982). Premature infants may have low or absent cysteinesulfinate decarboxylase (EC 4.1.1.29) activity and increased renal taurine losses (Helms et al, 1995). However, the differences in plasma taurine concentrations that were observed among diet treatments suggest that cysteinesulfinic acid decarboxylase activity is not limited in the neonatal piglet administered either enteral or parenteral nutrition. Furthermore, we observed much higher concentrations of plasma taurine than in our previous studies (Shoveller et al, 2003; Chapter 3.1, Bertolo et al, 2000, Elango et al, 2002). This supports the interpretation that the excess supply of cysteine resulted in the concomitant increased synthesis of taurine.

In conclusion, dietary cysteine can spare the methionine requirement for optimal protein synthesis by approximately 40% in both parenterally and enterally fed piglets. Comparison of the current data with piglets fed methionine alone showed that cysteine is not a dietary essential amino acid in neonatal piglets. Furthermore, in the presence of excess cysteine, the parenteral requirement for methionine is 31% lower than the enteral

requirement and this difference due to route of feeding is similar to our previous estimate when no cysteine was provided (Shoveller et al, 2002; Chapter 3.1).

3.2.5 **Literature Cited**

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3.3 THE BALANCE OF DIETARY SULFUR AMINO ACIDS AND THE ROUTE OF FEEDING AFFECT PLASMA HOMOCYSTEINE CONCENTRATIONS IN NEONATAL PIGLETS

3.3.1 Introduction

Hyperhomocysteinemia is an independent risk factor for cardiovascular and atherosclerotic disease in adults (Refsum et al, 1998). It is also associated with an increased risk of ischemic and hemorrhagic stroke in newborn infants and children (Hogeveen et al, 2002, Van Beynum et al, 1999). Reference values for plasma total homocysteine in human infants are between 5-8 μmol/L (Hogevenn et al, 2002, Bohles et al, 1999, Hongsprabhas et al, 1999). There is substantial evidence that blood values of plasma total homocysteine are often greater during deficiencies of vitamins B-6, B-12 and folate in adults and infants (Minet et al, 2000, House et al, 1999). Ambrosi and colleagues (1999) showed that 4.5 month-old pigs fed a methionine rich, casein based diet for one month developed hyperhomocysteinemia, which led to arterial lesions and thrombotic events. To our knowledge, no one has previously investigated the relationship between plasma total homocysteine concentration and dietary methionine intake in neonatal animals. The neonatal pig may be a useful model to study the relationship between hyperhomocysteinemia and stroke in infants and children.

There is currently great variation in the absolute concentration and ratio of sulfur amino acids in enteral infant formulas (Agonstoni et al, 2000) and breast milk (Forsum and Lonnerdal, 1979, Clark et al, 1987). Whether these differences effect plasma total homocysteine concentrations is not known. The dietary recommendation for adults,

children and infants with hyperhomocysteinemia is to supplement with folate and vitamin B-12 (Hogeveen et al, 2002). However, there are few data on how the dietary supply of the sulfur amino acids affect plasma total homocysteine concentrations in neonates with adequate intake of vitamins B-6, B-12, and folate. The potential effects of the differences in sulfur amino acid supply and ratio on plasma total homocysteine concentrations and also on long term health need to be determined.

Little information exists on how total parenteral nutrition (TPN) and the dietary concentration and ratio of sulfur amino acids in TPN affect plasma total homocysteine concentrations. TPN fed neonates have lower plasma cysteine concentrations than enterally fed neonates (Shoveller et al, 2003a (Chapter 3.2), Van Goudoever et al, 1995, Miller et al, 1995, Moss et al, 1999). Much like enteral formulas, there is great variation in the absolute concentrations of the sulfur amino acids and the methionine: cysteine ratio in currently available TPN solutions (Brunton et al, 2000). The clinical goal of both TPN and enteral feeding in the neonate is to provide sufficient nutrients to support rapid growth while avoiding potentially harmful excesses. One such deleterious effect may occur with an inappropriate ratio of methionine: cysteine, subsequently altering plasma total homocysteine and cysteine concentrations. Rabbits that were either TPN fed or chow fed, with supplemental intravenous methionine, had higher plasma total homocysteine concentrations than chow fed controls (Moss et al, 1999). In addition, TPN and chow fed rabbits receiving intravenous methionine treatments had decreased bile flow and hepatobiliary function; these events precede cholestasis, a common complication in TPN fed patients (Moss et al, 1999). Altering the methionine to cysteine ratio may alleviate the potentially harmful effects of high methionine concentrations in

TPN. Therefore, it is necessary to define the sulfur amino acid requirements of TPN fed neonates and understand how the intake and ratio of these amino acids affect plasma total homocysteine concentrations.

We have previously established the methionine requirements of parenterally and enterally fed piglets with (Shoveller et al, 2003a; Chapter 3.2) and without dietary cysteine (Shoveller et al, 2003b; Chapter 3.1). These data demonstrated that the gut is utilizing approximately 30% of the sulfur amino acids, that dietary cysteine can spare the methionine requirement by 40% in both routes of feeding and that cysteine is not a dietary indispensable amino acid in both enterally and parenterally fed neonatal piglets. We also measured plasma total homocysteine concentrations in samples obtained during these studies. The objective in the present study was to examine the effect of dietary methionine and cysteine intake on plasma total homocysteine concentrations in the parenterally and enterally fed neonatal piglet. Dietary cysteine decreases transmethylation and increases remethylation of methionine (DiBuono et al, 2003, Fukagawa et al, 1996) and thereby decreases plasma total homocysteine concentrations overall. We hypothesized that plasma total homocysteine would be positively associated with methionine intake in both parenterally and enterally fed piglets, but would be lower in diets that provide cysteine.

3.3.2 **Material and methods**

3.3.2.1 ***Animals and study protocol.***

The Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee at the University of Alberta approved all procedures used in this

study. A total of 53 male and 7 female Landrace/Large White piglets (Genex Swine Group) were obtained from the University of Alberta, Swine Unit (Edmonton, AB, CAN) at 1 to 2 d of age and at weights of 1.4 to 1.7 kg. The piglets underwent surgical implantation of venous catheters as previously described (Shoveller et al, 2003b; Chapter 3.1).

3.3.2.2 ***Animal housing.***

The piglets were housed and cared for as described in Shoveller et al (2003b; Chapter 3.1).

3.3.2.3 ***Diets.***

The piglets received 15 g amino acids $kg^{-1} \cdot d^{-1}$ and 1.1 MJ metabolizable energy $kg^{-1} \cdot d^{-1}$ with glucose and lipid (Intralipid 20%, Fresenius-Kabi, Stockholm, Sweden) each supplying 50% of nonprotein energy intake. The base amino acid profile of the complete elemental diet has been previously described (Shoveller et al, 2003b; Chapter 3.1). Vitamins were supplied in a commercial solution (3 mL per 900 mL of complete diet, Multi-12K_1 Pediatric, Sabex, Boucherville, PQ) that was added to the diet prior to feeding. The cofactors involved in methionine metabolism, vitamin B-12, B-6 and folate were provided by the MVI solution at approximately 115% of requirement (NRC, 1998). Piglets received a trace mineral solution that provided minerals at 200% of requirement (NRC, 1998), as previously described (Shoveller et al, 2003b; Chapter 3.1).

TPN was initiated immediately following surgery, and both enteral and parenteral groups were increased to full infusion rates (13.5 $mL \cdot kg^{-1} \cdot h^{-1}$) TPN and lipid) by the end of d 1. Complete TPN was continued until 1800 on d5. Piglets were then randomly allocated to one of the test levels of methionine with either no dietary cysteine or excess

dietary cysteine (0.55 g· kg^{-1}· d^{-1}). This concentration of dietary cysteine is 10% above the NRC (1998) recommendation for total sulfur amino acids and the increments of dietary methionine ranged between 5 and 200% of the total sulfur amino acid recommended intake (NRC, 1998). The dietary methionine intake varied between 0.025 and 1.0 g· kg^{-1}· d^{-1}. All test diet solutions were made isonitrogenous by balancing the test concentration of L-methionine with an increased or decreased in L-alanine concentration. Due to the highly unstable nature of L-cysteine in aqueous solutions, test diets were prepared immediately prior to infusion of the diet. The piglets were fed the test diet from 1800h on d5 until 1800h on d6. Blood was sampled at approximately 1400h on d6 (or 20h after test diet initiation). Subsequently, piglets were returned to the complete diet for 24h. At 1800h on d7, piglets were randomly assigned to a second test diet and blood was again sampled at approximately 1400 on d8 (or 20h on a second test diet). Piglets were then killed by injection of 1000 mg of sodium pentobarbital into a venous catheter.

3.3.2.4 ***Blood collection.***

Blood samples were collected into heparinized syringes via the femoral catheter. Whole blood samples were centrifuged at 4000 x g for 5 min and plasma was removed and frozen with liquid nitrogen and stored at -80°C until further analysis.

3.3.2.5 ***Determination of plasma total homocysteine.***

Plasma total homocysteine concentrations (protein bound + free) were analyzed according to the reverse phase-HPLC method of Araki and Sako (1987), with modifications as suggested by Gilfix et al. (1997). Briefly, plasma samples were incubated with tris-carboxyethylphosphine (Pierce Chemicals, Mississauga, ON), to

reduce protein-bound and oxidized forms of homocysteine, followed by derivatization with 7-fluorobenzofurazan-4-sulfonic acid ammonium salt (SBD-F; Sigma Chemical Co., Oakville, ON). The fluorescent thiol derivatives were separated on a Waters C-18 column (5 µM, 4.5 x 250 mm; Waters Canada, Mississauga, ON), using isocratic elution (98% 0.1 M acetate, pH 5.5: 2% methanol) by means of a Shimadzu HPLC system (Man-Tech Associates, Guelph, ON) complete with autoinjector and fluorescence detector (excitation λ = 385 nm; emission λ = 515 nm). Concentrations of total homocysteine were determined through the use of an external standard curve, and the inter- and intra-assay coefficients of variation were < 2%. This is a commonly used and well supported analytical method to determine total homocysteine concentrations (Pfeiffer et al, 2000).

3.3.2.6 ***Statistical analyses***.

The day of sampling (d6 or d8) and weight at study were determined not to be significant ($P>0.05$) using an ANOVA (SAS/STAT, version 8.1, SAS Institute, Cary, NC). Regression analysis variables were dietary intake of MET as the independent variable and plasma total homocysteine as the dependent variable. Slopes were tested for differences between among groups (GraphPad Prism, version 3.00 for Windows, GraphPad Software, San Diego California, USA). The slope of the regression line was not different among groups; therefore, differences between the y-intercepts of the regression lines for route of infusion and diet treatment (with or without dietary cysteine) were determined by pdiff analyzed as a 2 x 2 factorial using route of infusion and cysteine inclusion as the independent variables (SAS/STAT, version 8.1, SAS Institute, Cary, NC). The y-intercept represents the plasma total homocysteine concentrations.

3.3.3 **Results**

All piglets were healthy and active during the course of the study. Piglet weight upon arrival (2 d, 1645 g, pooled SD= 149) and weight at sampling (9 d, 2661 g, pooled SD= 297) were not significantly different among diet levels or route of feeding. Mean daily gain, prior to test diet initiation (160 g/d, pooled SD= 34), was not significantly different among diet levels or routes of feeding.

3.3.3.1 ***Plasma total homocysteine concentrations.***

Plasma total homocysteine concentrations were significantly and linearly associated with methionine intake within each treatment group ($P<0.01$; **Figure 3.3.1** and **Table 3.3.1**). The slope of the line was not different among all treatments ($P>0.05$); however, the intercept was significantly higher for enterally fed piglets that were provided no cysteine (IG) than all other treatments (Table 3.3.1, $P<0.05$). The linear response of plasma total homocysteine was similar both below and above the dietary requirement for total sulfur amino acids (Figure 1; Shoveller et al, 2003a (Chapter 3.2), Shoveller et al, 2003b, (Chapter 3.1)).

3.3.4 **Discussion**

Plasma total homocysteine concentrations increased in a linear relationship with increases in methionine intake regardless of route of feeding or provision of cysteine (Figure 3.3.1 and Table 3.3.1). The slope of the line was not different among all treatment groups, suggesting that the regulation of plasma total homocysteine by methionine intake was not affected by route of feeding or cysteine intake. In addition, given that the response was similar in all treatments both below and above the previously

Figure 3.3.1: The relationship between plasma homocysteine and methionine intake in intravenously fed neonatal piglets receiving: A. no cysteine (IV), where the equation of the line is: y=25.39x + 4.29 (R^2 = 0.55, P<0.0001) and excess cysteine (IV+C), where the equation of the line is: y=26.99x + 3.86 (R^2 = 0.84, P<0.0001). Correlation between plasma cysteine and methionine intake in intragastrically fed neonatal piglets receiving. B. no cysteine (IG), where the equation of the line is: y = 30.42x + 9.94 (R^2 = 0.37, P = 0.0006), and excess cysteine (IG+C), where the equation of the line is: y= 17.64x + 5.14 (R^2 = 0.25, P = 0.009).

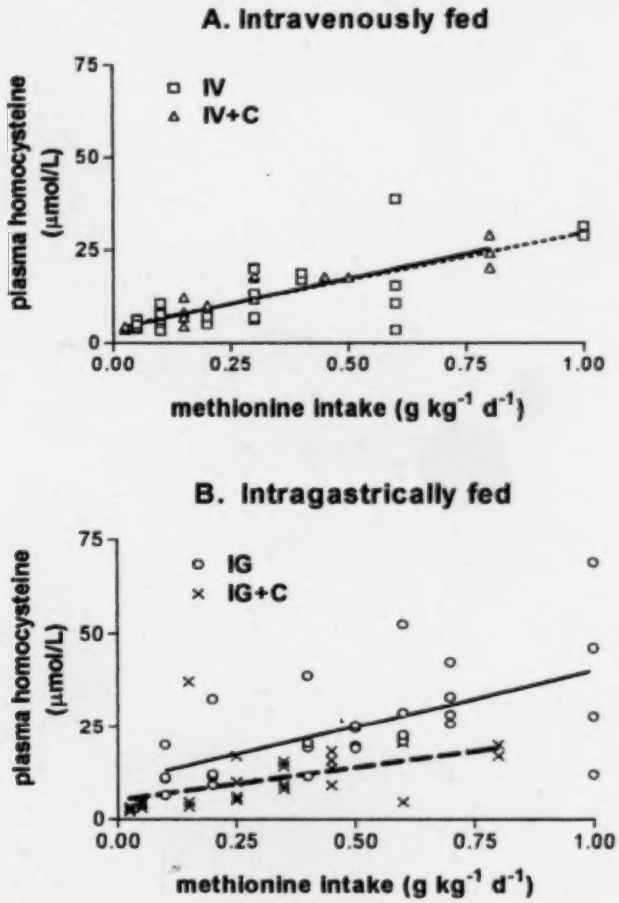

Table 3.3.1: Comparison of the slope and y-intercept among piglets intragastrically fed with (IG+C) or without cysteine (IG) and intravenously fed with (IV+C) or without cysteine (IV)[1].

Treatment	slope	intercept[2]	R^2	SE of the estimate	P value
IG+C	17.64	5.14[b]	0.25	1.39	0.009
IG	30.42	9.94[a]	0.37	2.22	0.0006
IV+C	26.99	3.86[b]	0.84	0.54	<0.0001
IV	25.39	4.29[b]	0.55	1.36	<0.0001
pooled values	30.65	N/A	0.50	0.82	<0.0001

[1]Values determined by regression analysis.
[2]Overall ANOVA, F-test, P<0.01. Values with different superscript letters indicate a significant difference among diet treatments as determined by pdiff in PROC GLM using a 2 x 2 factorial model with route of feeding and whether dietary cysteine was or was not supplied as the independent variables.

determined total sulfur amino acid requirement, the rate of protein synthesis, as measured by the oxidation of an indicator amino acid (Shoveller et al, 2003a (Chapter 3.2), Shoveller et al, 2003b, (Chapter 3.1)), did not affect the relationship between methionine intake and plasma total homocysteine concentration. The data reported herein are from studies designed to measure the methionine requirement after a ~20-h adaptation to a new dietary intake of methionine. Therefore, the changes we report here occurred within 20 h and indicate that the effects of methionine intake on plasma total homocysteine concentrations occur rapidly. In parenterally fed piglets, plasma total homocysteine concentrations were similar whether dietary cysteine was provided (IV+C) or not (IV), whereas during enteral feeding, the plasma total homocysteine concentrations were significantly higher when no dietary cysteine was provided (IG) than when dietary cysteine was provided (IG+C).

These data have implications for the provision of sulfur amino acids in enteral infant formulas because of the health risks associated with elevated plasma total homocysteine concentrations in newborns (Hogeveen et al, 2002) and children (Van Beynum et al, 1999). Currently, the ratio of methionine: cysteine in infant formulas varies depending on the protein source. Casein based formulas have a greater proportion of methionine, whereas both soy and whey based products provide less methionine and more taurine and few formulas contain additional cysteine (Agonstoni et al, 2000). Considering our findings, in a model that is relevant to nutrition in human infants (Brunton et al, 2000), sulfur amino acid intake and the ratio of the sulfur amino acids may have a profound effect on plasma total homocysteine concentrations. The question regarding optimum intake and ratio of the sulfur amino acids in formula fed infants and

the effects on plasma total homocysteine concentrations should be pursued further. In formulas where the majority of the sulfur amino acids are supplied by methionine, the addition of cysteine should be considered as a means of decreasing the plasma total homocysteine concentrations in infants fed the formula.

In the present study, all piglets received similar and more than adequate intakes of vitamin B-12, vitamin B-6 and folate and similar intakes of all other amino acids (NRC, 1998). Therefore, the increases in plasma total homocysteine we observed must be due solely to the methionine intake and not to differences in protein source or intakes of vitamins, nitrogen, amino acids or minerals. Other than methionine, the only dietary amino acid that was altered was alanine, which was used to make the diets isonitrogenous. The increase in plasma total homocysteine as dietary methionine increased suggests that the proportion of methionine that was transmethylated increased relative to the proportion that was transsulfurated and/or remethylated.

In the present study, the IG group had higher plasma total homocysteine concentrations at every level of methionine intake than the IG+C group. In adult men, the replacement of dietary methionine with cysteine (DiBuono et al, 2003) or glutathione (Fukagawa et al, 1996) reduced transmethylation of methionine to homocysteine and increased the remethylation of homocysteine to methionine. A reduction in transmethylation results in less homocysteine being synthesized whereas increased remethylation results in more homocysteine being remethylated to methionine. In total, the addition of dietary cysteine resulted in decreased homocysteine balance (appearance – disappearance) and therefore, decreased plasma total homocysteine, as observed in the present study. Regulatory processes similar to those in adult humans appear to be

present in piglets, as shown by the lower plasma total homocysteine concentrations in the IG+C group compared to the IG group. The regulatory effect of dietary cysteine on plasma total homocysteine does not exist in parenterally fed subjects, when the gut is bypassed. This is evidenced by the lack of difference in plasma total homocysteine concentrations whether cysteine was (IV+C) or was not provided (IV). The transmethylation/remethylation cycle is present in all cells (Finkelstein, 1998), so it is possible that the gut exports large amounts of homocysteine. However, given that oral dietary cysteine intake decreases transmethylation (DiBuono et al, 2003) and increases remethylation (DiBuono et al, 2003, Fukagawa et al, 1996); the addition of dietary cysteine may have partly mediated these effects within the gut.

Given that there was no difference in total homocysteine concentrations between IV and IV+C, we speculate that the gut is a larger site of homocysteine synthesis and release than the liver in neonatal piglets. However, adult rat liver incubated in methionine exported more homocysteine than hepatocytes incubated in methionine and cysteine (Stead et al, 2000). Further research is required to elucidate possible species and tissue differences in homocysteine metabolism. Alternatively, if cysteine utilization is greater during parenteral feeding, homocysteine balance may be reduced by the upregulation of the transsulfuration pathway in an attempt to maintain plasma cysteine concentrations. Indeed, plasma cysteine concentrations were lowest in the IV piglets, suggesting that there was increased utilization or catabolism of cysteine (Shoveller et al, 2003a; Chapter 3.2). Therefore, we speculate that luminal presence of cysteine results in less homocysteine exported from the gut compared to when no dietary cysteine is

provided and that parenterally fed piglets may require more cysteine than enterally fed piglets.

Given that the IG group had significantly higher plasma total homocysteine than the IG+C group, the whole body availability of cysteine may also play a role in homocysteine balance. Ventura et al (1999) found intravenous infusion of N-acetylcysteine, a cysteine precursor, resulted in increased urinary excretion of homocysteine in adults after 24 hours of intravenous N-acetylcysteine infusion (13.26 basal vs. 10.17 nmol mg^{-1}). Therefore, dietary cysteine may have increased the renal clearance and/or metabolism of homocysteine by cysteine displacing homocysteine from albumin binding sites and enabling homocysteine to be readily filtered by the kidney. This would result in a decrease in plasma total homocysteine; however, urinalysis was not conducted in the present experiment. Clearly, both the route of diet administration and the methionine: cysteine ratio play a regulatory role in sulfur amino acid metabolism and the regulation of this phenomenon needs to be further investigated.

Although meeting the total sulfur amino acid requirement for growth is important, supplying the correct ratio of methionine and cysteine, while avoiding high dietary methionine intakes is important. The present results agree with that of Rolland et al (1995) who demonstrated that mini-pigs fed a methionine-rich caseinate based diet developed hyperhomocysteinemia, establishing that pigs are a suitable model to study hyperhomocysteinemia. In addition, given the significantly higher plasma total homocysteine concentrations in the IG group compared to the IG+C group, supplying methionine as the sole sulfur amino acid source even at or below requirement may result in dangerously high plasma total homocysteine concentrations. If plasma total

homocysteine is directly related to an increased risk of ischemic and hemorrhagic stroke (Hogeveen et al, 2002, Van Beynum et al, 1999) our research implies that methionine intake in infants should be restricted. However, if methionine intake is restricted, cysteine supplementation is critical to maintain growth. Cysteine is unstable in aqueous solution; therefore, a cysteine precursor, such as N-acetylcysteine, could be supplemented to both enteral and parenteral diets to lower plasma homocysteine in the enterally fed infants and improve cysteine status in parenterally fed neonates. N-acetylcysteine may be an effective precursor for cysteine because it is stable in aqueous solution, highly bioavailable (Shoveller et al, 2002) and resistant to Maillard reactions (Baker and Han, 1993).

The present data clearly demonstrate that methionine intake, route of feeding and cysteine inclusion have a significant impact on plasma total homocysteine concentrations in neonatal piglets. The higher concentration of plasma total homocysteine in enterally fed piglets, given no dietary cysteine compared to those supplied dietary cysteine, has significant implications for the composition of infant formulas. Research into the role of splanchnic metabolism on homocysteine metabolism must be pursued using more complex in vivo techniques to measure sulfur amino acid kinetics under different dietary conditions and routes of feeding. Finally, we have shown that the neonatal piglet model is a sensitive model to investigate homocysteine metabolism in both the parenterally and enterally fed neonates.

3.3.5 **Literature cited**

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3.4 N-ACETYL-L-CYSTEINE IS A HIGHLY AVAILABLE PRECURSOR FOR CYSTEINE IN THE NEONATAL PIGLET RECEIVING TOTAL PARENTERAL NUTRITION

3.4.1 Introduction

The most effective method of supplying cysteine is a current issue in the design of neonatal total parenteral nutrition (TPN). Cysteine is widely accepted as a conditionally indispensable amino acid for the human infant. In premature infants there is low, but not absent, activity of hepatic cystathionase (EC 4.4.1.1), the rate limiting enzyme for the endogenous synthesis of cysteine from the indispensable amino acid methionine (Zlotkin et al, 1982). The activity of hepatic cystathionase increases after birth in both premature and term infants and is also present in the kidneys and adrenals (Zlotkin et al, 1982). A subsequent study in parenterally fed neonates reported that additional cysteine did not improve nitrogen retention compared to a parenteral solution containing no cysteine (Zlotkin et al, 1981). We have recently demonstrated in neonatal piglets that cysteine addition (0.55 g kg^{-1} d^{-1}) to both enteral and parenteral diets does not result in an improvement in whole body protein synthesis compared to diets that supplied methionine only (Shoveller et al, 2003a; Chapter 3.2). These data suggest that the neonate may be capable of adequate endogenous cysteine synthesis.

Other researchers have concluded that cysteine is indispensable in parenterally fed neonates because plasma concentrations of cysteine were extremely low (Van Goudoever et al, 1995, Pohlandt, 1974, Stegink and Den Besten, 1972). Indeed, we also

observed extremely low plasma concentrations of cysteine in parenterally fed neonatal piglets receiving no dietary cysteine (Shoveller et al, 2003a; Chapter 3.2). Further to this, plasma cysteine concentrations were significantly higher in parenterally fed piglets receiving cysteine supplemented total parenteral nutrition (Shoveller et al, 2003a; Chapter 3.2) and in human infants receiving total parenteral nutrition supplemented with cysteine-hydrochloride (Zlotkin et al, 1981) compared to those piglets and infants receiving no supplemental cysteine, respectively. Miller et al (1995) demonstrated that there was decreased cysteine synthesis in parenterally fed, premature infants using a stable isotope tracer of D-[U^{13}C]-glucose and measuring the enrichment of cysteine (from the conversion of glucose carbon into cysteine). Therefore, although methionine may meet the total sulfur amino acid requirement in infants fed total parenteral nutrition, cysteine supplementation may be required to normalize plasma total cysteine.

High methionine content in total parenteral nutrition has been shown to be hepatotoxic because it results in reduced bile flow and hepatobiliary function (Moss et al, 1999), which may lead to cholestasis. Although methionine can act as a substrate for glutathione synthesis, when given in excess, methionine depletes hepatic ATP (Hardwick et al, 1970) and raises plasma total homocysteine (Shoveller et al, 2004; Chapter 3.3). Further to this, hyperhomocysteinemia results in a reduction in adenosine reserves (Chen et al, 2002). This depletion of ATP and adenosine causes a shift of glutathione to its oxidized state (Corrales et al, 1991), which increases the liver's susceptibility to oxidative damage and may in part be responsible for cholestasis in parenterally fed neonates. Therefore, reducing the methionine content of total parenteral nutrition would reduce the occurrence of hepatotoxicity; however, if methionine intake is restricted,

cysteine supplementation becomes critical because of the need to satisfy the total metabolic requirement for the sulfur amino acids.

Cysteine supplemented total parenteral nutrition significantly increased plasma glutathione synthesis (Malloy and Rassin, 1984). Glutathione is a tri-peptide antioxidant that is endogenously synthesized. Premature infants have a lower rate of glutathione synthesis from methionine than from N-acetyl-L-cysteine (Vina et al, 1995) and this observation may partly explain why premature infants receiving total parenteral nutrition that is low in cysteine are highly susceptible to oxidative stress (Shahal et al, 1991). This increased glutathione synthesis may help to protect against oxidative stress in parenterally fed infants (Malloy and Rassin, 1984). More recently, Mosharov et al (2000) have reported that during oxidant stress, the transsulfuration pathway is upregulated, which leads to increased synthesis of glutathione *in vitro*, presumably from increased cysteine synthesis. These data suggest that additional cysteine should be supplied in neonatal parenteral formulas.

Despite the well accepted belief that cysteine is conditionally indispensable, few commercial parenteral solutions contain appreciable amounts of cysteine and others contain no cysteine at all; mainly due to the instability of cysteine in all heat sterilized solutions (Heird, 1998). Various forms of cysteine have been suggested for inclusion in parenteral solutions. Cysteine hydrochloride may be added to parenteral solutions, but has resulted in metabolic acidosis (Laine et al, 1991). Another approach is to supplement a stable precursor of cysteine that can be converted to cysteine *in vivo*.

N-acetyl-L-cysteine is a possible source of cysteine; however, its bioavailability has not been quantitatively determined. N-acetyl-L-cysteine has been shown to reduce

plasma levels of homocysteine by displacing thiol groups from their protein binding sites and forming sulfides that are rapidly excreted in the urine (Ventura et al, 1999). N-acetyl-L-cysteine is the most common orally and intravenously administered treatment for acetaminophen overdose (Yip et al, 1998) and as an antioxidant (Ahola et al, 1999). Acetaminophen is rapidly absorbed by the upper gastrointestinal tract and can reach toxic levels within 4 hours following an overdose (Compendium of Pharmaceuticals and Specialties, 2001). Although the majority of acetaminophen is non-toxic and metabolized in the liver to form sulfate and glucuronide conjugates, which are easily excreted in the urine, a small fraction forms a reactive, potentially toxic compound through cytochrome P-450 (Compendium of Pharmaceuticals and Specialties, 2001). This reactive and potentially toxic compound preferentially conjugates with hepatic glutathione to form a non-toxic cysteine and mercapturic acid derivative which is excreted by the kidney (Compendium of Pharmaceuticals and Specialties, 2001). Data examining the pharmacokinetics of intravenously administered N-acetyl-L-cysteine showed little correlation between N-acetyl-L-cysteine intake and plasma amino acid concentrations of cysteine (Ahola et al, 1999). N-acetyl-L-cysteine may provide a reserve pool of cysteine via increased glutathione or by replacing cysteine in mixed disulphides or cysteine that is bound to albumin. If N-acetyl-L-cysteine replaces cysteine in this fashion, it is reasonable to hypothesize that providing N-acetyl-L-cysteine will result in an increase in metabolically available cysteine. Furthermore, if N-acetyl-L-cysteine is a metabolically available source of cysteine, its supplementation to a diet deficient in total sulfur amino acids should increase growth, nitrogen retention and protein synthesis.

The null hypothesis of this research was that: N-acetyl-L-cysteine is not a bioavailable source of cysteine in the neonatal piglet receiving total parenteral nutrition. The objective of this research was to compare the effects of increasing N-acetyl-L-cysteine intake (0, 0.13 and 0.27 g kg^{-1} d^{-1} N-acetyl-L-cysteine) to a control, which provided 0.2 g kg^{-1} d^{-1} free base L-cysteine on measures of growth, nitrogen metabolism, N-acetyl-L-cysteine balance, sulfur amino acid metabolism and protein metabolism in neonatal piglets receiving total parenteral nutrition.

3.4.2 **Materials and Methods**

3.4.2.1 ***Animals and study protocol.***

The Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee at the University of Alberta approved all procedures in this study. A total of 20 male Landrace/Large White piglets (Genex Swine Group) were obtained from the University of Alberta, Swine Unit (Edmonton, AB, CAN) at 1-2 days old (1.5-1.9 kg). Piglets were transported to the Metabolic Unit at the University of Alberta. Upon arrival, the piglets were weighed and then anaesthetized with an intramuscular injection of acepromazine (0.5 mg/kg; Atravet™; Ayerst Laboratories, Montreal, PQ) and ketamine hydrochloride (22 mg/kg; Rogarsetic™; Rogar STB Inc., Montreal, PQ) and maintained during surgery with 1% halothane. All piglets were then fitted with venous catheters (Ed-Art, Don Mills, Canada) using the modified methods of Wykes et al. (1993). In all pigs, an infusion catheter was inserted into the left jugular vein and advanced to the superior vena cava just cranial to the heart and a sampling catheter was inserted into the left femoral vein and advanced to the inferior vena cava just caudal to

the heart. After surgery, incision sites were treated with a topical antiseptic (Hibitaine Veterinary Ointment: Ayerst Laboratories, Montreal, PQ) and an analgesic (0.1 mg/kg Buprenex, Buprenorphrine HCl, Reckitt and Colman Pharmaceutical Inc., Richmond, VA) was given intramuscularly. Analgesic was given again 8h-post surgery. Piglets were then put into cotton jackets, which secure a tether to the piglets. The tether is part of the swivel- tether system (Alice King Chatham Medical Arts, Los Angeles, California), that enables the pig to move freely while receiving a continuous dietary infusion and ensuring that the catheters to do not become tangled or occluded.

3.4.2.2 ***Animal housing.***

Animals were individually housed in metabolic cages, which were kept at a temperature of between 21°C-27°C using supplemental lamps for each individual cage. The lighting schedule was 12 h of light: 12 h of dark. Toys were added to enhance their environment.

3.4.2.3 ***Diet regimen.***

Elemental solutions were designed to meet the requirements of neonatal piglets (Wykes et al, 1993). Diets were administered continuously using pressure sensitive infusion pumps. Piglets received 15 g amino acids $kg^{-1} \cdot d^{-1}$ and 1.1 MJ metabolizable energy $kg^{-1} \cdot d^{-1}$ with glucose and lipid (Intralipid 20%, PharmaciaUpjohn, Stockholm, Sweden) each supplying 50% of nonprotein energy intake. The base amino acid profile of the complete elemental diet fed from day 0 until day 3 is presented as the control diet in **Table 3.4.1.** This amino acid profile is based on Vaminolact™, with the exceptions of phenylalanine, tyrosine and arginine. Phenylalanine and tyrosine were provided at their

Table 3.4.1: Amino acid concentrations of the total parenteral nutrition solutions administered to neonatal piglets during day 3 to 8.

Macronutrient	*Concentration (g/L)*
L-Alanine[1]	**zeroNAC:** 6.42 **lowNAC:** 6.15 **control and highNAC:** 5.88
L-Arginine	4.41
L-Aspartate	3.35
L-Cysteine[2]	**control: 0.74**
N-acetyl-L-cysteine	**zeroNAC:** 0 **lowNAC:** 0.50[6] **highNAC:** 0.99[7]
L-Glutamate	5.81
Glycine	0.44
L-Histidine	1.73
L-Isoleucine	2.54
L-Leucine	5.77
L-Lysine[3]	5.74
L-Methionine [4]	**1.10**
L-Phenylalanine	1.76
L-Proline	4.60
L-Serine	1.78
Taurine	0.26
L-Threonine	2.94
L-Tryptophan	1.18
L-Tyrosine	0
L-Valine	2.94
Glycyl-Tyrosine[5]	**2.01**

[1] Alanine was used to make the parenteral solution isonitrogenous.
[2] Cysteine was provided as L-cysteine free base.
[3] Lysine supplied as Lys-HCl.
[4] Methionine was maintained at 60% of total sulfur amino acid requirement for the parenterally fed piglet.
[5] Glycyl-tyrosine provided an additional 0.6334 g of Glycine and 1.5286 g of Tyrosine.
[6] N-acetyl-L-cysteine provided 0.36 g/L of free cysteine.
[7] N-acetyl-L-cysteine provided 0.74 g/L of free cysteine.

estimated safe levels of intake (House et al, 1997a, House et al, 1997b). Tyrosine was provided as the dipeptide glycyl-tyrosine (Wykes et al, 1994). Arginine was provided at 1.2 g kg^{-1} d^{-1} (Brunton et al, 1999). Vitamins were supplied in a commercial solution (Multi-12K_1 Pediatric, Sabex, Boucherville, PQ). The cofactors involved in the transsulfuration pathway, vitamin B_{12}, choline, B_6 and folate were in the MVI solution at approximately 115% of requirement (NRC, 1998). Piglets also received a mineral solution including zinc, copper, manganese, chromium, selenium and iodide at 200% of the NRC (NRC, 1998) recommendation for piglets. The L-cysteine used for the control diet was supplied as L-cysteine free-base (Degussa AG, 99% pharmaceutical grade). The N-acetyl-L-cysteine was provided by Fresenius-Kabi (batch # B0628, Certificate of Analysis in appendix A5).

All piglets received a amino acid and glucose/lipid (5:1) solution intravenously at 50% infusion rate for 12 hours beginning immediately following surgery and then 75% infusion rate until the morning after surgery (d1). Piglets were weighed daily and infusion rates were adjusted to provide nutrients on a per kg basis. On the morning of d1 total parenteral nutrition and lipid were increased to full infusion rate (13.5 mL kg^{-1} h^{-1}). All piglets received complete total parenteral nutrition (Table 3.4.1) until 600 h on d3. Piglets were then randomly allocated to one of the four dietary treatments: control (same as complete total parenteral nutrition), zeroNAC (0 g kg^{-1} d^{-1} NAC), lowNAC (0.13 g kg^{-1} d^{-1} NAC) and highNAC (0.27 g kg^{-1} d^{-1} NAC), Table 1) until the end of the flooding dose infusion on the morning of d8. LowNAC and highNAC diet treatments were isomolar to 0.10 and 0.20 g kg^{-1} d^{-1} cysteine and highNAC provided an equimolar amount of cysteine as the control treatment (0.2 g kg^{-1} d^{-1}). Methionine was provided at

0.30 g kg^{-1} d^{-1}, which we previously demonstrated was the upper 95% confidence interval of the methionine requirement when excess cysteine was provided to piglets receiving total parenteral nutrition (Shoveller et al, 2003a; Chapter 3.2). Therefore, total sulfur amino acid intake was limiting and if N-acetyl-L-cysteine is a biologically available precursor of cysteine, adding it to the total parenteral nutrition would improve overall growth and measures of amino acid and protein metabolism. All diets were made isonitrogenous by the addition or removal of L-alanine.

3.4.2.4 ***Urine collection.***

Beginning at 600 h on d3, urine was collected on ice four times daily and an aliquot was immediately frozen with liquid nitrogen and stored at -20°C until later analysis. Urine was measured for total nitrogen using the Kjeldahl method. Nitrogen balance was calculated as:

$$N_{balance}\ (g \cdot kg^{-1} \cdot d^{-1}) = N_{intake}\ (g \cdot kg^{-1} \cdot d^{-1}) - N_{output}\ (g \cdot kg^{-1} \cdot d^{-1})$$

Nitrogen retention was calculated as:

$$N_{retention}\ (\%) = N_{balance}\ (g \cdot kg^{-1} \cdot d^{-1}) / N_{intake}\ (g \cdot kg^{-1} \cdot d^{-1}) \times 100$$

Nitrogen output was equal to urinary nitrogen only. Stool outputs were negligible because all animals were intravenously fed and the collection period did not start until d3 post surgery.

3.4.2.5 ***Tissue collection.***

Blood samples were collected into heparinized syringes via the femoral catheter every 6 hours on d3 and then every 12 hours for the remainder of the study. Blood samples were centrifuged at 5000 x g for 5 minutes and plasma was frozen with liquid

nitrogen and then stored at -20°C during the sampling period (time required to sample all pigs) and then transferred to –80°C until further analyses.

On the morning of d8, piglets were infused with a flooding dose solution of unlabeled phenylalanine (150 mmol/L) in water containing 3700 MBq/L L-[H^3] phenylalanine (American Radiolabeled Chemicals, St. Louis, MO) for 5 minutes at a rate of 10 mL/kg body weight, resulting in a dose of approximately 37 MBq/kg of body weight. Blood samples were taken prior to the infusion and then at 5, 15 and 30 minutes post infusion initiation. After the 30 minute blood sample, piglets were anaesthetized with halothane and samples of liver, kidney, jejunum and muscle were excised and frozen with liquid nitrogen and stored at -80°C until later analyses. The small intestine was removed from the mesentery and its sheath. Jejunum (60 cm) was excised for mucosa collection, excluding the duodenum and the first ~10 cm of jejunum. The jejunal section was cut lengthwise and the mucosa was scraped off using a glass slide and even pressure, frozen in liquid nitrogen and stored at -80°C until further analyses. The time from the initiation of the flooding dose infusion to the freezing of each excised tissue was recorded accurately and considered in the calculation of protein synthesis. Piglets were then killed by lethal injection of 1000 mg of sodium pentobarbital.

3.4.2.6 ***Fractional and absolute protein synthetic rates.***

Fractional and absolute protein synthetic rates were measured using the flooding dose technique of Nyachoti et al (2000). 1 g of each tissue type was homogenized in 5 mL of 20 g/L of perchloric acid with a polytron and then centrifuged at 1500 X *g* for 15 min. The supernatant was recovered and kept frozen at - 20°C until later analysis. The

precipitate was washed twice with 8 mL of 1 mol/L sodium hydroxide and then left to stand in a water bath set at 37°C for 1.5 h to solubilize the proteins and a sample was taken. The solubilized protein was recovered by adding 4 mL of 200 g/L of cold perchloric acid and letting the mixture stand on ice for 20 minutes. The precipitated protein fraction was recovered by centrifugation at 2000 X *g* for 15 minutes followed by two washings with 8 mL of 20 g/L of perchloric acid.

The phenylalanine content of the precipitated protein pellet was determined following hydrolysis in 10 mL of 6 mol/L of hydrochloric acid in sealed, nitrogen flushed tubes at 110°C for 24h. The hydrolyzed samples were left to cool for 20 min and then thoroughly mixed before they were transferred into 125 mL Erlenmeyer flasks. Each tube was rinsed twice with deionized water that was added to their respective hydrosylates. The samples were then diluted to ~50 mL using deionized water and mixed thoroughly before filtering approximately 4 mL through a 0.22 µm filter with low protein binding ability (Millipore, Mississauga, Ontario, Canada). Phenylalanine concentrations in the hydrosylate, supernatant and plasma samples were determined according to the method of Bidlingmeyer et al (1984) which is described under *plasma and tissue amino acid concentrations*. Supernatant samples were analyzed in duplicate and hydrosylate samples were analyzed in triplicate. Thirty-five µL of each sample were injected onto the column (Picotag, Waters, Mississauga, Ontario, Canada). The phenylalanine peak was collected over a 3 min window, starting 1 minute prior and 1 min post elution time using a Waters Fraction Collector (Waters, Milford, MA). The radioactivity of the collected fractions was determined by a liquid scintillation counter after adding 10 mL of Biodegradable Counting Scintillant (Amersham Canada, Oakville,

Ontario, Canada). The background radioactivity was 8 dpm. All samples were counted for a minimum of 30 minutes or until the counting error was <2%.

Tissue protein content was measured according to Smith et al (1985) by the colormetric reaction with bicinchoninic acid (Sigma Chemicals, St. Louis, MO). The fractional rates of protein synthesis (FSR) were calculated using the method of Garlick et al (1983) using the following:

$$K_s\ (\%) = SRA_{bound} \times 100 / SRA_{free} \times time$$

where K_s is the fractional rate of protein synthesis as a percentage of the tissue protein pool synthesized per day, SRA_{bound} is the specific radioactivity of bound phenylalanine in the protein hydrosylates from each tissue, SRA_{free} is the specific radioactivity in the precursor pool and time refers to the amount of time available for the radiolabeled phenylalanine to incorporate into protein. Plasma phenylalanine SRA was measured in 5 randomly selected piglets to demonstrate that phenylalanine enrichment in plasma was at a steady state prior to tissue harvesting. Absolute synthetic rate was calculated using the following:

$$ASR\ (mg\ g^{-1}\ tissue\ d^{-1}) = (FSR\ (\%)/\ 100) * protein\ content\ of\ the\ tissue\ (mg/g).$$

3.4.2.7 ***Plasma, urine and tissue amino acids.***

All plasma, urine and tissue amino acids were measured using the method of Bidlingmeyer (1984) with modifications for biological samples according to House et al (1994). Detection limits for the amino acids was >1 mM. Plasma amino acids were determined using reverse phase-high performance liquid chromatography (HPLC). Briefly, a 200 µL sample are mixed with 40µL of 2.5 µmol/ L norleucine and 1 mL of a protein precipitant (0.5% triflouracetic acid (TFA): methanol) in 1.5 mL microcentrifuge

tubes and vortexed (Vortex; Fischer ScientificCo., Nepean, Ontario). Subsequently, samples were centrifuged at 5 000 x g for 5 minutes to remove the precipitated protein. After centrifugation, samples were transferred to 5mL plastic test tubes and frozen with liquid nitrogen and then freeze-dried (Lyph-Lock 4.5; Labconco, Fischer Scientific Co., Nepean, Ontario). Once dried, the samples were made alkaline by the addition of 50 µL of an amino acid elution solution, containing double deionized water (Milli-Q; Waters, Millipore Ltd., Milford, MA), methanol (HPLC grade; Fischer Scientific Co., Nepean, ON) and triethylamine (TEA; 99% pure; Alrich Chemical Co., Milwaukee, WI) in a 3:1:1 ratio. Samples were then vortexed and frozen with liquid nitrogen and freeze-dried. Once dried, the samples were derivatized by adding 20 µL of a solution containing purified water, methanol, phenylisothiocyanate (PITC) and TEA in a 1: 7: 1: 1 ratio. After a 35-minute derivatization period, the samples were frozen with liquid nitrogen and freeze-dried to ensure long-term stability.

For tissue free amino acids, 100µL of 25 mM norleucine was added to 100 mg of tissue, homogenized in a mixture of 10 mL trifluoroacetic acid and 100 mL methanol and centrifuged. For urine, 2.0 mL of sample was mixed with 100 µL of 25 mM norleucine and 2.0 mL of a precipitant (0.5 mL triflouroacetic acid/100 mL methanol) and centrifuged at 5000 x g for 5 minutes to remove any proteins. The mixture was then run through Dowex (50-8X) and running 5 mL of 2 M ammonium hydroxide through the column subsequently eluted the amino acids. The supernatant was used for further preparation of tissue free amino acids.

After the samples were dry, they were resuspended in 200µL of sample diluent of a 95% phosphate buffer: 5% acetonitrile (HPLC grade; Fischer Scientific Co., Nepean,

ON) solution and vortexed. Samples were then centrifuged and the aqueous layer was transferred to 0.5-mL microcentrifuge tubes. Samples were stored at –80 ^{O}C until analysis.

Amino acid derivatives were analyzed by reverse-phase HPLC. Aliquots of the derivatized samples were injected (WISP 710B™; Waters, Millipore Ltd., Milford, MA) onto a C^{18} column (Novapack™; Waters, Millipore Ltd., Milford, MA), heated to 46°C (TCM™; Waters, Millipore Ltd., Milford, MA). Amino acid derivatives in the injected samples were detected at a wavelength of 254 nm (Model 440 Absorbance Detector; Waters, Millipore Ltd., Milford, MA). Gradient control and peak integration was carried out by a computing software package (Maxima™; Waters, Millipore Ltd., Milford, MA).

Gradient chromatographic conditions were established between an aqueous salt buffer and an aqueous organic solvent elution buffer. The aqueous salt buffer was prepared by mixing (4 x 1000mL) of double deionized water (Milli-Q; Waters, Millipore Ltd., Milford, MA)and (4 x 9.5g) of sodium acetate, adjusting the pH to 6.55, removing 100 mL, then adding 100 mL of acetonitrile (HPLC grade; Fischer Scientific Co., Nepean, ON). The aqueous organic solvent buffer was prepared by mixing (4 x 400mL) of double deionized water (HPLC grade; Fischer Scientific Co., Nepean, ON), (4 x 450 mL) of acetonitrile (HPLC grade; Fischer Scientific Co., Nepean, ON) and (4 x 150 mL) of methanol (HPLC grade; Fischer Scientific Co., Nepean, ON). All prepared buffers were filtered before use to remove any gas within the mixture.

To calculate amino acid concentrations the following equation was used:

*[amino acid] (µmol/L) = (amino acid peak area / Norleucine peak area) * CF * RF*

where CF is the concentration factor (250 μmol/L) and RF is the response factor for that amino acid. Response factor was calculated as follows:

RF= Norleucine peak area / amino acid peak area

for equimolar standards.

3.4.2.8 ***Plasma urea nitrogen.***

Plasma urea concentrations were measured using a spectrophotometric assay kit in which ammonia is liberated from urea by enzymatic hydrolysis by urease (Sigma Chemical, St. Louis, MO).

3.4.2.9 ***Plasma ammonia.***

Plasma ammonia concentrations were determined for each blood sample from the start of test diet infusions. The spectrophotometric assay used in the analysis was based on the amination of 2-oxoglutarate to glutamate with simultaneous oxidation of NADPH (Sigma Procedure No. 171-UV, Sigma Diagnostics, St. Louis, MO).

3.4.2.10 ***Plasma and urine N-acetyl-L-cysteine.***

Plasma and urine N-acetyl-L-cysteine concentrations were determined by reverse-phase high-performance liquid chromatography using NBD-Cl derivatives as previously described (Frank and Thiel, 1984). Plasma and urine concentrations of N-acetyl-L-cysteine were determined using a standard curve that was run with every set of samples. Detection limits in the present study were 25 μM, which were slightly higher than those reported by Frank and Thiel (1984), which were 5 μM. Samples that were below 25 μM were considered to be zero.

Plasma samples (250 μL) were first deproteinized with 250 μL of methanol. The protein pellet was discarded and the supernatant was kept for further derivatization. Urine did not require deproteinization. 250 μL of sample (urine or deproteinized plasma) was then reduced by the addition of 50 μL of phosphate buffer (17.8 g Na_2HPO_4-$2H_20$ in 200 mL HPLC grade water, pH adjusted to 8.0 with phosphoric acid) and 10 μL of dithioerythritol (145 mg in 10 mL HPLC grade water). Samples were then vortexed and allowed to stand for 30 minutes. At 30 minutes, 250 μL of the sample was pipetted into a 500 μL eppendorf and 250 μL of EDTA/Citrate buffer (29.4 g of sodium citrate and 744 mg of EDTA dissolved in HPLC grade water, adjusted to pH 8.3 with sodium hydroxide) and 100 μL of NBD-Cl (100 mg of 4-Chloro-7-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl) in 100 mL of methanol) were added. Samples were vortexed and allowed to stand for 15 minutes. Samples were then centrifuged at 5000 x g for 5 minutes. After 20 minutes the sample was ready for chromatography. Standards were dissolved in HPLC grade water and treated the same as samples. Aliquots of 20 μL were injected onto the column. The mobile phase was 0.5% disodium hydrogen phosphate and acetonitrile (80:20, volume/volume) with a flow rate of 2.0 mL/min. The samples were monitored at 420 nm with a spectrophotometer. The column used was a C^{18} column (Novapack™; Waters, Millipore Ltd., Milford, MA) that was kept at a temperature of 30°C.

3.4.2.11 ***Statistical analyses.***

A completely randomized design, with dietary treatment serving as the main treatment effect was used in this experiment. If p values were < 0.05 for the F-value of

the ANOVA model (SAS/STAT, version 8.1, SAS Institute, Cary, NC), significant differences between treatments were determined using Student Newman Keul's multiple comparison procedure. For body and organ weight data, weight at initiation of test diet (d3) was used as a covariate because there were significant differences in body weight between groups on d3. For data in which there were repeated samples (plasma urea, plasma ammonia, nitrogen balance, N-acetyl-L-cysteine balance) data was analyzed using repeated measures ANOVA to determine if there was an effect of sampling times from initiation of test diet, on the dependent variable. If P values were <0.05 for the F value of the model, the slope of the response over time was considered to be significant and the shape of the response was defined. All data was graphed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

3.4.3 **Results**

Preliminary studies to verify tolerance to the total parenteral diets and develop and verify methods were conducted in March to June of 2001. The animal portion of this experiment, as described below, was conducted over four months starting in July 2001.

3.4.3.1 ***Body weight, average daily gain and organ weights.***

All piglets were healthy and active during the course of the study. Piglet weight upon arrival was 1.75 kg (pooled SD= 0.15). The day 3, 4, 5, 6, and 7 mean body weights (**Table 3.4.2**) were significantly higher in the zeroNAC, lowNAC and highNAC groups

Table 3.4.2: Daily mean body weight[1] (g) of piglets receiving different concentrations of N-acetyl-L-cysteine or a control parenteral solution.

day	control	zeroNAC	lowNAC	highNAC	P value	pooled SE
3*	1832[b]	2078[a]	2078[a]	2054[a]	<0.05	34
4[2]	1960[b]	2320[a]	2266[a]	2210[a]	<0.05	43
5[2]	2092[b]	2520[a]	2474[a]	2390[a]	<0.05	51
6[2]	2270[b]	2678[a]	2630[a]	2584[a]	<0.05	50
7[2]	2458[b]	2554[a]	2780[a]	2748[a]	<0.05	46
8[2]	2648	2772	2922	2928	NS[3]	52

[1]Values are means of N=5 piglets. Values are expressed as mean weight (g) and are taken during the test period (day 3 (600h) to day 8 (600h).
[2] Body weight on day 3 was used as a covariate because day 3 weights were significantly different.
[3] NS, not significant.
*Overall P values were determined by analysis of variance, using diet as the independent variable. Means with different superscripts within a row are significantly different at P<0.05 and were determined using Student Newman Keul's Multiple Comparisons procedure.

than the control group ($P<0.05$). On day 8, there were no differences among groups ($p>0.05$).

Because there were initial differences in body weight between treatment groups, average daily gain is represented as g/100g body weight to remove body weight as a factor and represent weight gain as a percentage of body weight. On days 4, 5 and 6 mean average daily gains were not significantly different among treatments (**Table 3.4.3**). On day 3, zeroNAC piglets had significantly higher gains than highNAC and control piglets ($p<0.05$), but were not different from lowNAC piglets. The day 7 average daily gains were similar among control, lowNAC and highNAC and zeroNAC exhibited the lowest average daily gains ($P<0.05$). Total gains for the experimental period were not significantly different among diet treatments. The effect of time on average daily gain showed a significant decrease in daily weight gain for zeroNAC ($P<0.0001$) and lowNAC piglets ($P=0.02$) and had no significant effect on either control or highNAC piglets.

There were no differences in organ weights at necropsy among the treatment groups (**Table 3.4.4**).

3.4.3.2 ***Water balance***.

In order to deliver sufficient nutrients to mimic the nitrogen intake of suckling piglets of similar ages, a fluid intake of ~ 290 ml/kg/d was targeted in the present study. We have previously shown that for the first 3 or 4 days this may result in fluid (water) retention (Wykes et al, 1993). Piglets receiving zeroNAC took longer to adapt to the fluid load than the other three treatments as shown by the higher water retention on day 3

Table 3.4.3: Mean average daily weight gain (g/100g BW)[1] of piglets receiving different dietary concentrations of N-acetyl-L-cysteine or a control diet.

Day	control	zeroNAC	lowNAC	highNAC	P value	pooled SE
3*	6.98[b]	11.64[a]	9.02[ab]	7.56[b]	0.01	0.60
4	6.80	8.58	9.10	8.16	NS[2]	0.48
5	8.54	6.32	6.28	8.22	NS	0.60
6	8.34	3.06	5.68	6.32	NS	0.80
7	7.76[a]	0.60[b]	5.00[a]	6.52[a]	0.02	0.89

[1]Values are means of N=5 piglets. Values are expressed as mean average daily gain (g/100g). Average daily gain is calculated as: (the difference between the present day's weight- the previous day's weight) divided by the previous day's weight. Average daily gain was calculated from body weights taken during the test period (day 3 (600h) to day 8 (600h).

[2] NS, not significant.

*Overall P values were determined by analysis of variance, using diet as the independent variable. Means with different superscripts within a row are significantly different at $P<0.05$ and were determined using Student Newman Keul's Multiple Comparisons procedure.

Table 3.4.4: Mean organ weights of piglets receiving different dietary concentrations of N-acetyl-L-cysteine or a control diet[1].

	control	zeroNAC	lowNAC	highNAC	p value	pooled SE
body weight (g)	2686	2771	2930	2936	NS[2]	50.86
liver weight (g)	145.16	133.52	130.62	138.98	NS	3.07
Kidney weight (g)	23.04	24.36	24.28	23.66	NS	0.84
Jejunum weight (g)	4.88	5.06	4.65	4.80	NS	0.12

[1]Values are means of N=5 piglets. Measurements were taken on the morning of day 8 at necropsy. There were no significant differences among treatments.
[2] NS, not significant.

(**Table 3.4.5**). After this initial difference, there were no differences in water retention (mL kg^{-1} d^{-1}) among treatments groups.

3.4.3.3 ***Plasma and urinary nitrogen analyses.***

All treatments had plasma ammonia concentrations within the normal range (12-120 μmol/L; Brunton et al, 1999). This demonstrates that the restriction in dietary sulfur amino acids was not so severe that it caused a disturbance in the disposal of urea from the catabolism of excess dietary amino acids. There was no effect of time on plasma ammonia.

Plasma urea was measured to indicate the adequacy of the amino acid supply. Normal range of plasma urea nitrogen is 3-5 mmol/L (Brunton et al, 1999). ZeroNAC piglets had the lowest concentration of dietary sulfur amino acids and we observed significantly higher plasma urea nitrogen in these piglets (8.14 ± 1.00 mmol/L; $P<0.05$) than for all other treatment groups. As the intake of N-acetyl-L-cysteine increased, plasma urea decreased. In the lowNAC treatment plasma urea was 4.89 ± 0.51 mmol/L. The lowest levels of plasma urea were observed in the control (1.82 ± 0.36 mmol/L) and highNAC treatments (2.62 ± 0.22 mmol/L; $P<0.05$). Over the 5-day experimental period, plasma urea significantly increased ($P<0.05$) in the zeroNAC and lowNAC treatments (**Figure 3.4.1**). Plasma urea in control and highNAC piglets did not increase during the experimental period ($P>0.05$).

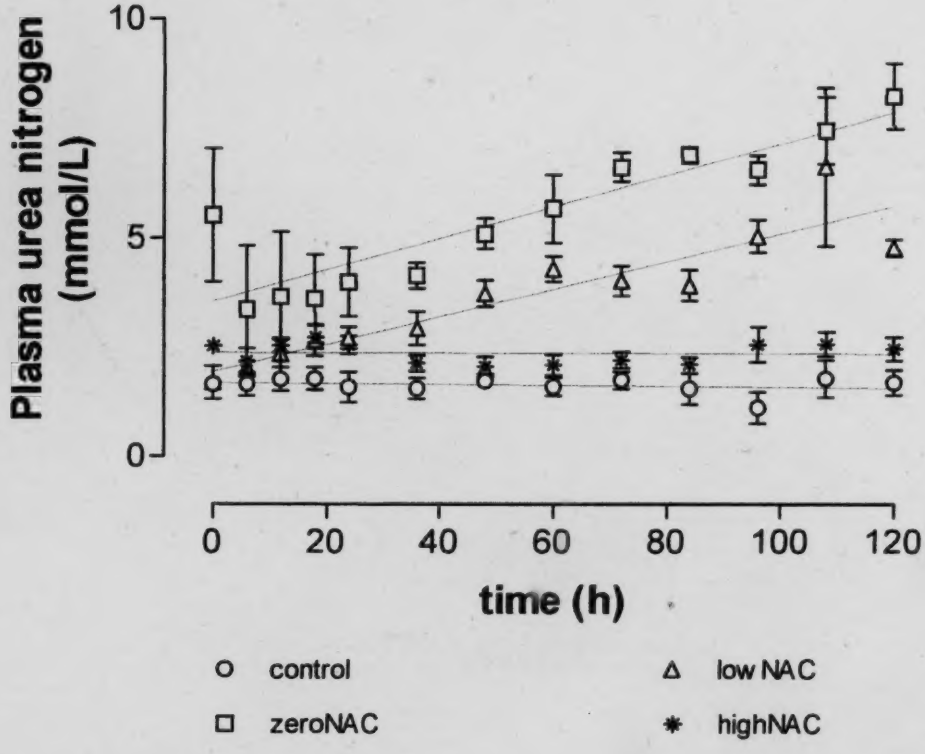
Nitrogen intake was not different among any diet treatments. Because no faecal outputs were detected, due to the intravenous feeding, urinary excretion of nitrogen was considered to be total output. ZeroNAC piglets had the lowest nitrogen retention and

Table 3.4.5 : Mean water retention[1] (mL kg BW^{-1} day^{-1}) of piglets receiving different dietary concentrations of N-acetyl-L-cysteine or a control diet.[2]

day	control n=5	zeroNAC n=5	lowNAC n=5	highNAC n=5	P value	pooled SE
3*	52.99[b]	132.80[a]	88.74[b]	74.21[b]	<0.05	8.96
4	72.94	81.21	91.54	89.01	NS[3]	4.47
5	34.38	66.99	67.75	89.99	NS	12.94
6	92.55	52.54	65.19	89.04	NS	7.10
7	79.55	88.85	69.67	71.29	NS	8.30
average (mL/kg/d)	66.42	84.48	76.61	82.71	NS	4.37
total (mL)	332.11	422.39	383.03	413.53	NS	21.85

[1]Values are expressed as mean water retention (mL/kg/day) = (intake volume (mL) – urine volume (mL))/ BW (kg). Values were taken from measurements taken during the test period (day 3 (600h) to day 8 (600h).
[2]Insensible water losses (respiratory and transepidermal) were not measured but assumed to be similar among groups.
[3]NS, not significant.
*Significantly different ($P<0.05$, Student Newman Keul's). Letters indicate differences among dietary treatments.

Figure 3.4.1 : Plasma urea concentrations of piglets receiving different dietary concentrations of N-acetyl-L-cysteine or a control diet. Time 0 represents day 3 at 600h, immediately prior to test diet initiation. Each data point is presented as mean ± SEM for N=5 piglets. The y-intercept for the zeroNAC group was significantly higher than the lowNAC group ($P<0.05$). Over the five-day experimental period, plasma urea concentrations significantly increased ($P<0.0001$) in both zeroNAC and lowNAC. There was no effect of time on plasma urea in both highNAC and control treatments. The equation of the regression line for zeroNAC was: $y= 0.036x + 3.56$ ($R2 = 0.81$); for lowNAC was: $y=0.031x + 1.95$ ($R2 = 0.85$); for highNAC was: $y = - 0.0003x + 2.39$ ($R2 = 0.04$) and for control was: $y = 0.001x + 1.69$ ($R2 = 0.06$).



lowNAC piglets were intermediate between both highNAC and control treatments which were not different from each other (**Table 3.4.6**; $P<0.05$). In the zeroNAC and lowNAC treatments there was a significant decrease in daily nitrogen retention over the five-day treatment period (**Figure 3.4.2**; $P<0.05$). A decrease in nitrogen retention over time requires an increase in nitrogen excretion over time; this explains the rising plasma urea concentrations observed in these two treatments.

3.4.3.4 ***Plasma and urine N-acetyl-L-cysteine.***

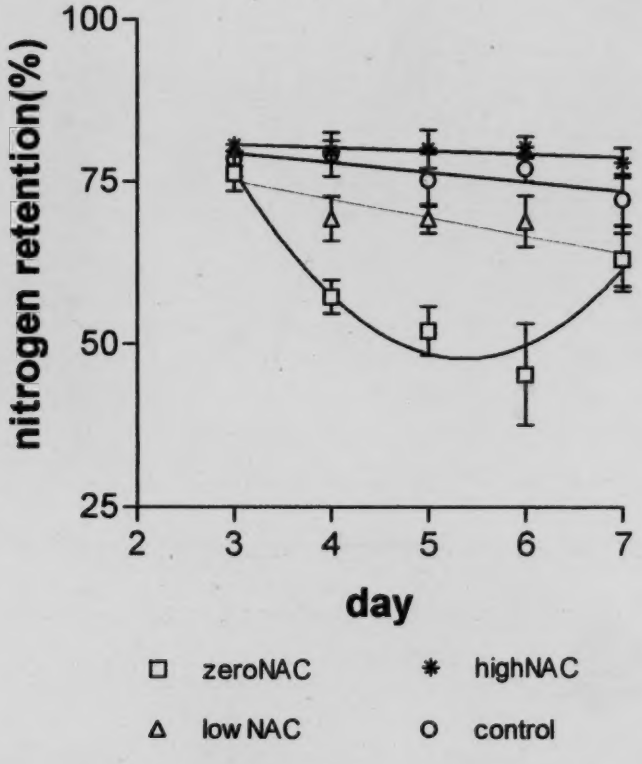
Daily N-acetyl-L-cysteine retention was calculated as dietary intake minus urinary output divided by dietary intake. The urine of zeroNAC and control piglets was measured but N-acetyl-L-cysteine was either below detectable limits (~ 0.025mM) or was not present; therefore, the N-acetyl-L-cysteine concentration in the urine of these pigs was considered to be zero. N-acetyl-L-cysteine intakes were significantly different between groups and resulted in values very close to predicted intakes of 0.13 and 0.27 g kg^{-1} day^{-1} for both dietary treatments (**Table 3.4.7**). N-acetyl-L-cysteine output in urine was lower and the amount retained (g kg^{-1} d^{-1}) was higher for highNAC ($P<0.05$) in comparison to lowNAC piglets. ZeroNAC and control had zero or undetectable concentrations of N-acetyl-L-cysteine. N-acetyl-L-cysteine retention (% of intake) was not significantly different between lowNAC and highNAC (Table 3.4.7) indicating that increasing intake did not significantly affect the degree of bioavailability. N-acetyl-L-cysteine retention showed a significant linear decline ($P=0.02$) over time and this response was not different between lowNAC and highNAC (Table 3.4.A17). Although there was a significant response in N-acetyl-L-cysteine retention over time, the time x diet interaction was not significant.

Table 3.4.6: Nitrogen balance of piglets receiving different dietary concentrations of N-acetyl-L-cysteine or a control diet[1].

	control	zeroNAC	lowNAC	highNAC	P value	pooled SE
Nitrogen intake (g/kg/day)	2.160	2.158	2.150	2.148	0.86	0.005
Nitrogen output* (g/kg/day)	0.508^{c}	0.888^{a}	0.654^{b}	0.432^{c}	<0.0001	0.044
Nitrogen retained (g/kg/day)	1.651^{a}	1.270^{c}	1.494^{b}	1.714^{a}	<0.0001	0.045
Nitrogen retention (%)	76.40^{a}	58.77^{c}	69.49^{b}	79.80^{a}	<0.0001	2.08

[1]Values are means of N=5 piglets. Values are expressed as nitrogen retention (%) = (nitrogen intake-nitrogen output)/nitrogen intake x 100%. Values are an average of 5 days of collection taken during the test period (day 3 (600h) to day 8 (600h).
*Overall P values were determined by analysis of variance, using diet as the independent variable. Means with different superscripts within a row are significantly different at $P<0.05$ and were determined using Student Newman Keul's Multiple Comparisons procedure.

Figure 3.4.2 : Daily nitrogen retention of piglets receiving different dietary concentrations of N-acetyl-L-cysteine. Each data point is presented as mean ± SEM for N=5 piglets. Over the five-day experimental period, nitrogen retention significantly decreased ($P<0.05$) in both zeroNAC and lowNAC. Daily nitrogen retention did not change significantly in highNAC and control piglets over the 5 day treatment period. The equation of the regression line for zeroNAC was: $y= 195.7 -55.04x + 5.121x^2$ ($R^2 = 0.99$); for lowNAC was: $y= -2.779x + 83.4$ ($R^2 = 0.82$); for highNAC was: $y = -0.492x +82.14$ ($R^2 = 0.51$) and for control was: $y = -1.47x + 83.75$ ($R^2 = 0.69$).



* Day represents the 24 hour collection period from 6am the day indicated until 6am the following day for the entire test period.

Table 3.4.7 : N-acetyl-L-cysteine balance of piglets receiving different dietary concentrations of N-acetyl-L-cysteine or a control diet[1].

	control	zeroNAC	lowNAC	highNAC	pooled SE[¥]
N-acetyl-L-cysteine intake* (g/kg/day)	0^{c}	0^{c}	0.128^{b}	0.266^{a}	0.023
N-acetyl-L-cysteine output (g/kg/day)	0^{c2}	0^{c2}	0.019^{b}	0.0525^{a}	0.0059
N-acetyl-L-cysteine retained (g/kg/day)	0^{c}	0^{c}	0.1099^{b}	0.2135^{a}	0.019
N-acetyl-L-cysteine retention (%)	0	0	85.39	82.61	1.73

[1]Values are means of N=5 piglets. Values for each piglet were averaged for day 3 through day 7 as measurements taken during the test period (day 3 (600h) to day 8 (600h).

[2]N-acetyl-L-cysteine could not be detected in the urine from the control and zeroNAC piglets. Detection limit was <0.025 mM.

*Overall P values were determined by analysis of variance, using diet as the independent variable. Means with different superscripts within a row are significantly different at $P<0.05$ and were determined using Student Newman Keul's Multiple Comparisons procedure.

[¥] Pooled standard error presented represents the variation observed in lowNAC and highNAC diet groups only.

Plasma N-acetyl-L-cysteine was significantly higher in the highNAC group than the lowNAC group (**Figures 3.4.3 and 3.4.4**). Over the five-day experimental period, plasma N-acetyl-L-cysteine concentrations did not change in the lowNAC group ($P>0.05$), but significantly increased over the five-day experimental period in the highNAC group ($P<0.05$). N-acetyl-L-cysteine was not detected in the plasma of control or zeroNAC piglets.

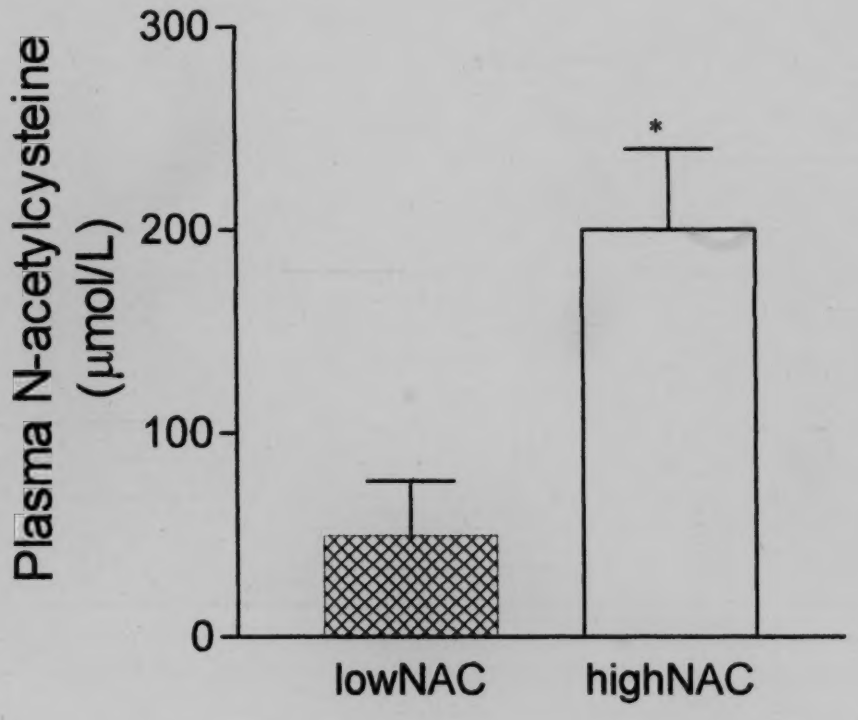
3.4.3.5 ***Plasma, urine and tissue amino acid concentrations***.

Plasma methionine concentrations were highest in the control treatment, which was not different from highNAC but was higher than zeroNAC and lowNAC treatments (**Table 3.4.8**: $P<0.05$). A similar effect was observed for threonine (Table 3.4.8). Conversely, plasma glycine concentrations were similar among zeroNAC, lowNAC and highNAC groups ($P>0.05$). All treatments had higher plasma glycine concentrations than the control group (Table 3.4.8: $P<0.05$). Plasma serine was significantly higher in the zeroNAC treatment and all other treatments were not different (Table 3.4.8: $P<0.05$). All other amino acids did not differ between treatments (Table 3.4.8: $P>0.05$).

Daily urinary excretion of taurine (**Table 3.4.9**) and methionine (**Table 3.4.10**) were similar among groups ($P>0.05$). Urinary cystine concentrations were higher in the control piglets, which received L-cysteine free base, than all other treatments for the entire duration of the study (**Table 3.4.11**: $P<0.05$).

Concentrations of free amino acids in the liver are presented in **Table 3.4.12**. Methionine, tyrosine, lysine, leucine, isoleucine, valine, tryptophan, proline, citrulline, ornithine and glutamate were all lower in the control treatment than all N-acetyl-L-cysteine treatments ($P<0.05$). Glycine, aspartate, glutamine, phenylalanine, taurine and

Figure 3.4.3 : Mean plasma N-acetyl-L-cysteine concentrations for both lowNAC and highNAC treatments. Each bar represents the mean ± SEM for N=5 piglets for a period of 5 days (n=13 samples per diet treatment). Plasma N-acetyl-L-cysteine concentrations were significantly higher for the highNAC piglets than the lowNAC piglets ($P<0.05$, paired t-test). Detection limit for N-acetyl-L-cysteine was 0.025 mM.



*** denotes significantly different using a paired t-test assuming equal variances between diet treatments ($P<0.05$).**

Figure 3.4.4 : Time course of plasma N-acetyl-L-cysteine concentrations over the 120-h (5-day) test period for lowNAC and highNAC treatments. N-acetyl-L-cysteine was undetectable in control and zeroNAC piglets. Each data point is presented as mean ± SEM for N=5 piglets. The y-intercept for the zeroNAC treatment was significantly lower than the lowNAC treatment ($P<0.05$). Over the five-day experimental period, plasma N-acetyl-L-cysteine concentrations did not change in the lowNAC group ($P>0.05$), but significantly increased over the five-day experimental period in the highNAC group ($P<0.05$). The equation of the linear regression line for highNAC was: $y = -1.69x + 91.08$ ($R2 = 0.60$, P value = 0.002) and the second order polynomial line was: $y = 51.60 + 4.116x - 0.03x2$ ($R2 = 0.93$, P value <0.01).

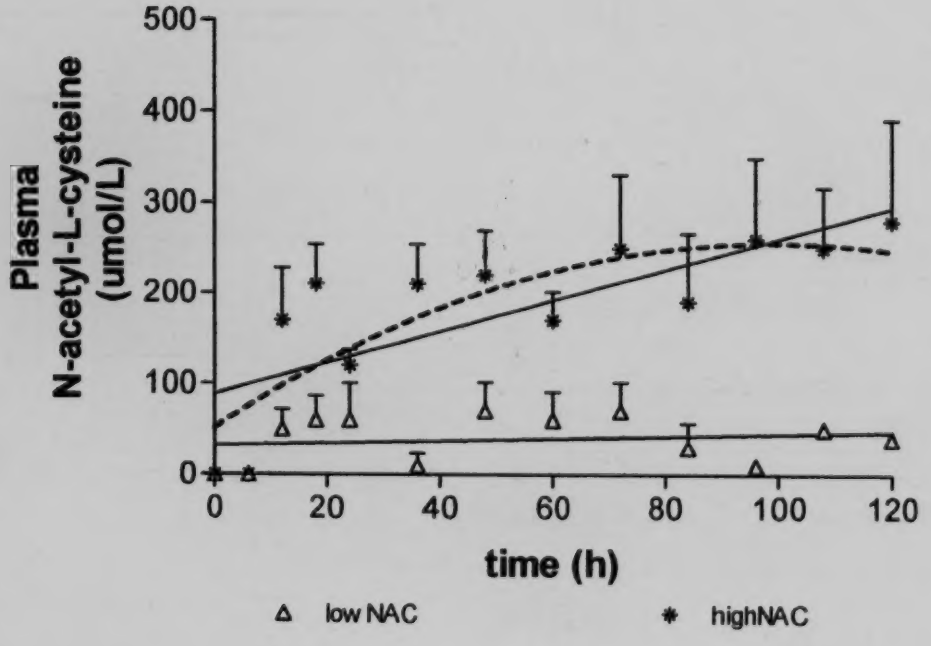


Table 3.4.8: Plasma free amino acid concentrations of piglets receiving different dietary concentrations of N-acetyl-L-cysteine or a control diet after a five-day adaptation to test diets[1]

amino acid	control	zeroNAC	lowNAC	highNAC
methionine[2]	36 ± 7^{a}	9 ± 2^{b}	9 ± 1^{b}	29 ± 7^{a}
cystine	14 ± 5	5 ± 4	1 ± 1	5 ± 2
taurine	129 ± 17	164 ± 14	139 ± 17	132 ± 17
serine	438 ± 38^{b}	742 ± 34^{a}	517 ± 89^{b}	509 ± 84^{b}
phenylalanine	28 ± 3^{c}	95 ± 14^{a}	56 ± 6^{b}	70 ± 32^{ab}
tyrosine	41 ± 6^{b}	143 ± 33^{a}	100 ± 12^{ab}	71 ± 18^{ab}
lysine	496 ± 54	553 ± 54	449 ± 33	463 ± 46
leucine	127 ± 19	196 ± 21	190 ± 7	395 ± 196
isoleucine	58 ± 16	103 ± 11	92 ± 12	177 ± 84
valine	126 ± 20	227 ± 26	192 ± 14	275 ± 104
threonine	395 ± 70^{ab}	109 ± 17^{c}	184 ± 23^{bc}	471 ± 145^{a}
histidine	80 ± 18	118 ± 26	86 ± 13	121 ± 35
arginine	105 ± 12	88 ± 16	89 ± 3	189 ± 85
proline	581 ± 77	922 ± 165	787 ± 82	759 ± 185
citrulline	40 ± 6	38 ± 8	62 ± 7	37 ± 16
ornithine	97 ± 20	91 ± 11	72 ± 24	115 ± 15
glutamate	237 ± 37	208 ± 26	164 ± 21	448 ± 182
glutamine	160 ± 21	179 ± 49	264 ± 9	204 ± 36
alanine	431 ± 48	604 ± 101	449 ± 32	788 ± 322
aspartate	33 ± 5	53 ± 8	53 ± 8	138 ± 97
hydroxyproline	56 ± 7	49 ± 5	54 ± 4	73 ± 15
glycine	724 ± 66^{c}	1504 ± 70^{a}	1270 ± 102^{ab}	1037 ± 200^{ab}

[1]Values are means of N=5 piglets. Values represent means ± SE. Values are from measurements taken at necropsy on day 8.

[2]Overall P values were determined by analysis of variance, using diet as the independent variable. Means with different superscripts within a row are significantly different at $P<0.05$ and were determined using Student Newman Keul's Multiple Comparisons procedure.

Table 3.4.9 : Daily urinary taurine (μmol)[1] excretion by piglets receiving different dietary concentrations of N-acetyl-L-cysteine or a control diet[1].

Taurine (μmol)	control	zeroNAC	lowNAC	highNAC	pooled SE
Day 3	67	900	119	146	186
Day 4	201	613	78	78	105
Day 5	171	339	127	946	218
Day 6	91	412	177	169	63
Day 7	148	706	532	575	162

[1]Values are means of N=5 piglets. Measurements are from samples taken during the test period (day 3 (600h) to day 8 (600h).

Table 3.4.10 : Daily urinary methionine (μmol)[1] excretion by piglets receiving different dietary concentrations of N-acetyl-L-cysteine or a control diet.

Methionine (μmol)	control	zeroNAC	lowNAC	highNAC	pooled SE
Day 3	11	15	13	9	1
Day 4	13	19	23	14	3
Day 5	19	25	21	12	3
Day 6	18	21	18	14	1
Day 7	20	16	27	11	2

[1]Values are means of N=5 piglets. Measurements are from samples taken during the test period (day 3 (600h) to day 8 (600h).

Table 3.4.11: Urinary cystine (μmol)[1] excretion of piglets receiving different dietary concentrations of N-acetyl-L-cysteine or a control diet. Numbers represent total daily excretion.

Cystine (μmol)	control	zeroNAC	lowNAC	highNAC	pooled SE
Day 3[2]	40^{a}	15^{b}	11^{b}	11^{b}	4
Day 4	65^{a}	9^{b}	37^{ab}	9^{b}	7
Day 5	79^{a}	10^{b}	11^{b}	10^{b}	8
Day 6	74^{a}	7^{b}	17^{b}	9^{b}	7
Day 7	90^{a}	5^{b}	10^{b}	9^{b}	9

[1]Values are means of N=5 piglets. Measurements are from samples taken during the test period (day 3 (600h) to day 8 (600h).
[2]Overall ANOVA, F-test, $P<0.01$. For data in a row, different superscript letters indicate a significant difference between diet treatments within day ($P<0.05$).

Table 3.4.12 : Liver free amino acid concentrations of piglets receiving different dietary concentrations of N-acetyl-L-cysteine or a control diet after a five-day adaptation to test diets[1]

(nmol/g wet tissue)	control	zeroNAC	lowNAC	highNAC
Methionine[2]	294 ± 26^{b}	727 ± 139^{a}	722 ± 129^{a}	781 ± 164^{a}
Cystine	83 ± 42^{a}	20 ± 8^{b}	11 ± 2^{b}	14 ± 8^{b}
Taurine	5045 ± 691^{a}	766 ± 204^{b}	883 ± 179^{b}	2488 ± 1215^{b}
Serine	2542 ± 378	4766 ± 790	4276 ± 204	3774 ± 412
Phenylalanine	1450 ± 99^{a}	1184 ± 116^{b}	1137 ± 49^{b}	1324 ± 50^{ab}
Tyrosine	154 ± 22^{b}	978 ± 190^{a}	823 ± 143^{a}	676 ± 229^{a}
Lysine	931 ± 96^{b}	1654 ± 118^{a}	1477 ± 199^{a}	1410 ± 175^{a}
Leucine	843 ± 88^{b}	1872 ± 303^{a}	1947 ± 322^{a}	1925 ± 343^{a}
Isoleucine	326 ± 30^{b}	738 ± 128^{a}	703 ± 121^{a}	694 ± 113^{a}
Valine	619 ± 59^{b}	1304 ± 168^{a}	1203 ± 188^{a}	1194 ± 194^{a}
Threonine	1053 ± 84	829 ± 130	930 ± 133	1162 ± 123
Histidine	327 ± 46	353 ± 20	352 ± 28	363 ± 40
Tryptophan	10 ± 3^{b}	90 ± 19^{a}	98 ± 19^{a}	94 ± 21^{a}
Arginine	114 ± 28	111 ± 6	78 ± 9	122 ± 16
Proline	1246 ± 137^{b}	2656 ± 276^{a}	2510 ± 311^{a}	2357 ± 389^{a}
Citrulline	233 ± 15^{b}	968 ± 251^{a}	1325 ± 254^{a}	1273 ± 280^{a}
Ornithine	342 ± 36^{b}	987 ± 117^{a}	851 ± 125^{a}	815 ± 122^{a}
Glutamate	2243 ± 130^{b}	3434 ± 280^{a}	3330 ± 181^{a}	2779 ± 292^{ab}
Glutamine	1832 ± 222^{a}	1191 ± 276^{ab}	879 ± 394^{b}	631 ± 231^{b}
Alanine	3221 ± 235	4204 ± 407	3748 ± 225	3992 ± 431
Asparagine	859 ± 88	839 ± 88	5060 ± 1818	5057 ± 1865
Aspartate	3717 ± 235^{a}	2676 ± 474^{b}	3227 ± 425^{ab}	3067 ± 85^{ab}
Hydroxyproline	96 ± 28	61 ± 20	53 ± 14	61 ± 16
Glycine	10214 ± 256^{a}	8566 ± 1300^{ab}	4281 ± 2418^{b}	3732 ± 1773^{b}

[1]Values are means of N=5 piglets. Values represent means ± SE. Values are from measurements taken at necropsy on day 8.

[2]Overall P values were determined by analysis of variance, using diet as the independent variable. Means with different superscripts within a row are significantly different at $P<0.05$ and were determined using Student Newman Keul's Multiple Comparisons procedure.

cystine were higher in the control than all other N-acetyl-L-cysteine treatments ($P<0.05$). Liver concentrations of all other amino acids did not differ between treatments.

Concentrations of free amino acids in the jejunum are presented in **Table 3.4.13**. Methionine, histidine, arginine and ornithine were lower in the jejunum of control piglets than all other treatments ($P<0.05$). Cystine, taurine, glutamate and hydroxy-proline concentrations were higher in the control piglets than all other treatments ($P<0.05$). Serine and tryptophan were highest in the lowNAC piglets and did not differ from lowNAC and highNAC treatments. The lowest concentrations of serine and tryptophan were found in the control treatment ($P<0.05$). Valine and alanine concentrations were highest in the zeroNAC and highNAC treatments ($P<0.05$), which did not differ from the lowNAC piglets, but were higher than the control treatment. All other amino acid concentrations did not differ between treatments ($P>0.05$).

Kidney free amino acids are presented in **Table 3.4.14**. Methionine and tryptophan concentrations were higher in highNAC piglets than control and zeroNAC treatments ($P<0.05$). Cystine, leucine, isoleucine, valine, histidine and glutamate concentrations were similar in control, lowNAC and highNAC treatments, which were higher than the zeroNAC treatment ($P<0.05$). Taurine, alanine, and aspartate concentrations were highest in the control group ($P<0.05$). Phenylalanine concentrations were lowest in the control treatment than all other treatments ($P<0.05$). Tyrosine concentrations were highest in the control, which was not different from highNAC but greater than both zeroNAC and lowNAC ($P<0.05$). Citrulline and glycine concentrations were highest in the control treatment, but lower in the highNAC

Table 3.4.13 : Jejunum free amino acid concentrations of piglets receiving different dietary concentrations of N-acetyl-L-cysteine or a control diet after a five-day adaptation to test diets[1]

(nmol/g wet tissue)	control	zeroNAC	lowNAC	highNAC
Methionine[2]	111 ± 21^{b}	455 ± 82^{a}	424 ± 93^{a}	510 ± 112^{a}
Cystine	37 ± 5^{a}	12 ± 10^{b}	8 ± 7^{b}	10 ± 7^{b}
Taurine	4125 ± 333^{a}	2141 ± 762^{b}	2308 ± 676^{ab}	2267 ± 667^{ab}
Serine	930 ± 251^{b}	1467 ± 109^{a}	1296 ± 99^{ab}	1401 ± 73^{ab}
Phenylalanine	1080 ± 210	1158 ± 57	1016 ± 53	1158 ± 55
Tyrosine	1951 ± 687	1268 ± 626	1024 ± 325	1135 ± 283
Lysine	681 ± 21	927 ± 124	807 ± 88	926 ± 120
Leucine	628 ± 59	1215 ± 145	988 ± 108	1139 ± 124
Isoleucine	306 ± 27	378 ± 67	357 ± 30	393 ± 21
Valine	433 ± 97^{b}	844 ± 120^{a}	703 ± 73^{ab}	808 ± 82^{a}
Threonine	600 ± 142	529 ± 108	375 ± 96	542 ± 44
Histidine	151 ± 17^{b}	462 ± 77^{a}	561 ± 97^{a}	615 ± 115^{a}
Tryptophan	11 ± 7^{b}	33 ± 8^{a}	30 ± 3^{ab}	28 ± 7^{ab}
Arginine	310 ± 115^{b}	1348 ± 185^{a}	1377 ± 282^{a}	1417 ± 289^{a}
Proline	599 ± 245	1153 ± 303	847 ± 238	521 ± 311
Citrulline	162 ± 29	194 ± 25	186 ± 17	219 ± 13
Ornithine	100 ± 23^{b}	222 ± 22^{a}	198 ± 21^{a}	210 ± 26^{a}
Glutamate	1495 ± 110^{a}	369 ± 328^{b}	317 ± 257^{b}	965 ± 613^{b}
Glutamine	1330 ± 883	777 ± 103	814 ± 140	901 ± 153
Alanine	1273 ± 324^{b}	2615 ± 309^{a}	1770 ± 375^{ab}	2267 ± 180^{a}
Asparagine	619 ± 134	501 ± 46	417 ± 51	431 ± 28
Aspartate	2733 ± 301	2834 ± 142	2862 ± 231	2677 ± 258
Hydroxyproline	116 ± 8^{a}	54 ± 23^{b}	45 ± 11^{b}	49 ± 11^{b}
Glycine	ND[3]	2908 ± 774	3909 ± 365	3922 ± 313

[1]Values are means of N=5 piglets. Values represent means ± SE. Values are from measurements taken at necropsy on day 8.

[2]Overall P values were determined by analysis of variance, using diet as the independent variable. Means with different superscripts within a row are significantly different at $P<0.05$ and were determined using Student Newman Keul's Multiple Comparisons procedure.

[3] ND is an abbreviation for not determined.

Table 3.4.14 : Kidney free amino acid concentrations of piglets receiving different dietary concentrations of N-acetyl-L-cysteine or a control diet after a five-day adaptation to test diets[1]

(nmol/g wet tissue)	control	zeroNAC	lowNAC	highNAC
Methionine[2]	196 ± 13^{b}	134 ± 15^{b}	281 ± 50^{ab}	407 ± 96^{a}
Cystine	34 ± 3^{a}	15 ± 4^{b}	33 ± 3^{a}	44 ± 7^{a}
Taurine	6949 ± 262^{a}	2299 ± 700^{b}	2786 ± 574^{b}	3819 ± 1275^{b}
Serine	1378 ± 111	925 ± 176	1202 ± 116	1404 ± 209
Phenylalanine	454 ± 37^{b}	691 ± 76^{ab}	740 ± 102^{ab}	981 ± 182^{a}
Tyrosine	1796 ± 169^{a}	340 ± 89^{b}	657 ± 253^{b}	1011 ± 514^{ab}
Lysine	662 ± 60	645 ± 166	663 ± 87	825 ± 161
Leucine	873 ± 69^{a}	286 ± 18^{b}	749 ± 97^{a}	993 ± 174^{a}
Isoleucine	445 ± 33^{a}	122 ± 16^{b}	353 ± 49^{a}	456 ± 82^{a}
Valine	810 ± 58^{a}	214 ± 26^{b}	586 ± 82^{a}	749 ± 129^{a}
Threonine	682 ± 53	422 ± 244	468 ± 64	632 ± 103
Histidine	363 ± 27^{a}	83 ± 10^{b}	379 ± 105^{a}	264 ± 44^{a}
Tryptophan	65 ± 19^{a}	29 ± 5^{b}	43 ± 7^{ab}	62 ± 11^{a}
Arginine	511 ± 29	623 ± 527	311 ± 36	404 ± 64
Proline	1781 ± 140	1187 ± 477	1338 ± 147	1513 ± 217
Citrulline	502 ± 21^{a}	116 ± 23^{c}	192 ± 51^{bc}	288 ± 77^{b}
Ornithine	137 ± 12	128 ± 38	178 ± 25	243 ± 61
Glutamate	2213 ± 227^{a}	1041 ± 297^{b}	2195 ± 108^{a}	2713 ± 138^{a}
Glutamine	573 ± 42	723 ± 605	414 ± 52	524 ± 97
Alanine	2840 ± 142^{a}	1846 ± 209^{b}	1906 ± 171^{b}	2277 ± 170^{b}
Asparagine	718 ± 52^{a}	160 ± 15^{c}	443 ± 127^{b}	596 ± 97^{ab}
Aspartate	5623 ± 604^{a}	1409 ± 256^{b}	2131 ± 890^{b}	2418 ± 879^{b}
Hydroxyproline	314 ± 26^{a}	123 ± 18^{c}	206 ± 24^{b}	257 ± 39^{ab}
Glycine	7359 ± 399^{a}	3360 ± 445^{c}	4659 ± 574^{bc}	5116 ± 537^{b}

[1]Values are means of N=5 piglets. Values represent means ± SE. Values are from measurements taken at necropsy on day 8.

[2]Overall P values were determined by analysis of variance, using diet as the independent variable. Means with different superscripts within a row are significantly different at $P<0.05$ and were determined using Student Newman Keul's Multiple Comparisons procedure.

treatment, which was not different than the lowNAC treatment. The lowest concentrations of citrulline and glycine were found in the zeroNAC group ($P<0.05$). Asparagine and hydroxyproline were highest in the control treatment, which was not different than highNAC, but higher than the lowNAC treatment and the lowest concentrations were found in the zeroNAC treatment ($P<0.05$). All other amino acids did not differ between treatment groups ($P>0.05$).

Muscle (longissimus dorsi) free amino acids are presented in **Table 3.4.15**. Taurine, tyrosine, histidine, tryptophan, proline, citrulline, glutamine, alanine and glycine concentrations were greater in control than all other treatments ($P<0.05$). Methionine, serine, lysine, threonine, arginine, ornithine, asparagine and hydroxyproline concentrations were affected similarly (Table 3.4.15). Glutamate was highest in the highNAC treatment, which did not differ from the zeroNAC and lowNAC treatments but was greater than the control treatment ($P<0.05$). All other amino acids did not differ between treatment groups ($P>0.05$).

Fractional synthetic rates were not different between treatment groups (**Table 3.4.16**: $P>0.05$) using an ANOVA to compare fractional synthetic rate (FSR) and absolute synthetic rate (ASR) among dietary treatments. Kidney FSR ($P=0.04$, $R^2=0.33$) and ASR ($P=0.05$, $R^2=0.29$) were positively and linearly associated with increasing N-acetyl-L-cysteine intake (Table 3.4.16). Neither liver nor gastrocnemius showed a significant effect of N-acetyl-L-cysteine intake (Table 3.4.16) on fractional or absolute synthetic synthesis, although numerical increases, with increases in N-acetyl-L-cysteine intake were observed.

Table 3.4.15 : Longissimus dorsi free amino acid concentrations of piglets receiving different dietary concentrations of N-acetyl-L-cysteine or a control diet after a five-day adaptation to test diets[1]

(nmol/g wet tissue)	control	zeroNAC	lowNAC	highNAC
Methionine[2]	144 ± 13^{a}	75 ± 16^{b}	102 ± 27^{ab}	90 ± 10^{ab}
Cystine	7 ± 3	2 ± 2	10 ± 10	3 ± 2
Taurine	5757 ± 148^{a}	1308 ± 396^{b}	3017 ± 1174^{b}	3032 ± 725^{b}
Serine	1353 ± 190^{a}	959 ± 42^{ab}	726 ± 157^{b}	1069 ± 128^{ab}
Phenylalanine	590 ± 47	747 ± 54	521 ± 101	704 ± 139
Tyrosine	531 ± 122^{a}	133 ± 48^{b}	208 ± 131^{b}	134 ± 67^{b}
Lysine	1199 ± 195^{a}	273 ± 62^{c}	486 ± 56^{bc}	633 ± 94^{b}
Leucine	154 ± 34	104 ± 5	130 ± 32	139 ± 16
Isoleucine	49 ± 8	60 ± 5	62 ± 18	60 ± 7
Valine	128 ± 10	108 ± 16	153 ± 43	132 ± 18
Threonine	882 ± 76^{a}	383 ± 93^{bc}	253 ± 54^{c}	497 ± 40^{b}
Histidine	146 ± 11^{a}	27 ± 6^{b}	48 ± 32^{b}	32 ± 11^{b}
Tryptophan	41 ± 8^{a}	10 ± 8^{b}	7 ± 5^{b}	8 ± 7^{b}
Arginine	3289 ± 167^{a}	1086 ± 169^{c}	1904 ± 459^{b}	3408 ± 225^{a}
Proline	2847 ± 413^{a}	789 ± 105^{b}	1179 ± 281^{b}	1315 ± 250^{b}
Citrulline	370 ± 14^{a}	53 ± 27^{b}	121 ± 61^{b}	140 ± 85^{b}
Ornithine	216 ± 45^{a}	34 ± 10^{b}	57 ± 19^{b}	173 ± 27^{a}
Glutamate	1192 ± 140^{b}	1610 ± 136^{ab}	1575 ± 334^{ab}	1983 ± 174^{a}
Glutamine	2196 ± 268^{a}	148 ± 49^{b}	689 ± 310^{b}	1026 ± 392^{b}
Alanine	3346 ± 247^{a}	1331 ± 225^{b}	1535 ± 460^{b}	1910 ± 288^{b}
Asparagine	153 ± 22^{a}	ND[3]	51 ± 36^{ab}	34 ± 24^{b}
Aspartate	2825 ± 72^{a}	203 ± 53^{b}	749 ± 446^{b}	826 ± 383^{b}
Hydroxyproline	138 ± 15^{a}	41 ± 8^{c}	74 ± 14^{bc}	110 ± 19^{ab}
Glycine	5944 ± 333^{a}	3461 ± 843^{b}	2606 ± 947^{b}	2501 ± 666^{b}

[1]Values are means of N=5 piglets. Values represent means ± SE. Values are from measurements taken at necropsy on day 8.

[2]Overall P values were determined by analysis of variance, using diet as the independent variable. Means with different superscripts within a row are significantly different at $P<0.05$ and were determined using Student Newman Keul's Multiple Comparisons procedure.

[3]ND is the abbreviation for not detectable.

Table 3.4.16 : Estimates of fractional and absolute synthetic rates of *in vivo* protein synthesis in liver, kidney and gastrocnemius of piglets receiving different dietary concentrations of N-acetyl-L-cysteine or a control diet[1].

	control	zeroNAC	lowNAC	highNAC	P value	SE	Linear effect[4] P value
Liver							
Fractional synthetic rate (% d^{-1})[2]	82.16	85.50	98.25	91.27	0.73	5.52	0.63
Absolute synthetic rate (mg g^{-1} d^{-1})[3]	54.32	49.05	52.37	53.67	0.98	4.75	0.69
Kidney							
Fractional synthetic rate (% d^{-1})	94.92	49.53	74.62	86.55	0.12	7.06	0.04
Absolute synthetic rate (mg g^{-1} d^{-1})	51.31	19.33	33.09	39.58	0.09	4.41	0.05
Gastrocnemius							
Fractional synthetic rate (% d^{-1})	75.07	55.97	75.79	99.98	0.40	11.63	0.13
Absolute synthetic rate (mg g^{-1} d^{-1})	57.51	25.67	30.91	42.24	0.21	6.73	0.17

[1]Values are means ± SD of N=5 piglets. Values are from measurements taken at necropsy on day 8.
[2] Fractional synthetic rates are calculated as: FSR =(SRA_{bound}/SRA_{free}) x (1440 minutes/labeling time) x 100%
[3]Absolute synthetic rate is calculated as: ASR = (FSR/100) x protein content of the tissue (mg/g tissue)
[4]Linear effect assessed by linear regression (Graphpad Prism). The control group was not included in this analysis. The linear regression was used to examine the effect of increasing dietary N-acetyl-L-cysteine.

3.4.4. **Discussion**

In enteral diets, methionine and cysteine comprise the total sulfur amino acids and are generally supplied in a 50:50 (weight: weight) ratio in the diet. However, no commercial parenteral solutions contain an appreciable amount of cysteine. Cysteine supplementation to parenteral solutions would increase whole body availability of cysteine and presumably increase glutathione concentrations, which would help to avoid the toxic effects of a high methionine diet (Moss et al, 1999). However, cysteine is unstable in parenteral solutions and will quickly oxidize to its dimer, cystine, which is poorly soluble. Therefore, cysteine's instability must be overcome to avoid the toxic effects of methionine, increase the utilization of all other amino acids for protein synthesis and increase plasma cysteine and presumably glutathione concentrations. To effectively supply cysteine in solution the precursor must be soluble, stable in solution and effectively converted to cysteine *in vivo*. N-acetyl-L-cysteine is not only soluble and stable, it is also resistant to Maillard destruction (Baker et al, 1984), thus enabling heat treatment of the solution. Therefore, this study was designed to evaluate N-acetyl-L-cysteine as a precursor for cysteine in neonates receiving total parenteral nutrition, using the well-established and widely accepted piglet model.

The present study demonstrates that N-acetyl-L-cysteine was as effective as an equimolar amount of L-cysteine in supporting growth and tissue protein synthesis, as evidenced by equal growth rates (Table 3.4.2 and 3.4.3), low blood urea nitrogen (Figure 1), high nitrogen balance (Table 3.4.6) and high protein accretion (Table 3.4.16). In addition, N-acetyl-L-cysteine retention was high (Table 3.4.7) demonstrating that approximately 85% of infused N-acetyl-L-cysteine was retained. Together these data

demonstrate that an equimolar intake of N-acetyl-L-cysteine can support growth and nitrogen retention that is not significantly different from free L-cysteine. The present findings agree with Neuhauser et al (1986) who demonstrated that rats receiving total parenteral nutrition that supplied methionine as the sole source of the sulfur amino acids had similar nitrogen balance and growth as rats that had partial replacement of methionine by N-acetyl-L-cysteine (on a molar basis). Furthermore, only 4.6% of the total infused N-acetyl-L-cysteine was detected in the urine of these rats, suggesting that N-acetyl-L-cysteine was approximately 95% available. Baker and Han (1993) found that young rats fed a casein based oral diet supplemented with either L-cysteine or N-acetyl-L-cysteine achieved similar growth rates.

In adult humans receiving a 4-hour intravenous infusion of N-acetyltyrosine or N-acetyl-L-cysteine, 56% and 11% of the infused amount was detected in the urine, respectively (Magnusson et al, 1989). These authors' estimate of the urinary excretion of N-acetyltyrosine (56%) is similar to our measurement of 65% excretion of N-acetyltyrosine in neonatal piglets receiving total parenteral nutrition (Wykes et al, 1994). We determined the bioavailability of N-acetyltyrosine with similar techniques as in the present study (Wykes et al, 1994). The present estimate of 15% urinary excretion of N-acetyl-L-cysteine (Table 3.4.7) is similar to the 11% urinary excretion determined by Magnusson et al (1989). However, Magnusson et al (1989) supplied a very high dose of N-acetyl-L-cysteine, suggesting that the acylases were not overwhelmed and N-acetyl-L-cysteine has a low toxicity. Unfortunately, these researchers did not report plasma concentrations of the other sulfur containing amino acids, specifically taurine and methionine, which may have provided additional evidence of utilization.

In contrast, VanGoudoever et al (1994) compared three different amino acid solutions, one (Aminovenos, Fresenius AG) of which contained a low (5 mg/ g protein) concentration of N-acetyl-L-cysteine while the other solutions contained free base L-cysteine, administered to premature neonates for 5 days. These researchers found more than 50% of the infused N-acetyl-L-cysteine was excreted in the urine and plasma cystine concentrations remained low. The intake of N-acetyl-L-cysteine in the lowNAC treatment (7 mg/g amino acids) in the present study is slightly higher than the intake given by Van Goudoever et al (5 mg/g amino acids); however, the plasma N-acetyl-L-cysteine concentrations in the lowNAC treatment were ~ 40 μM, which is higher compared to Van Goudoever et al (1994),which were 18 μM. The dietary supply of N-acetyl-L-cysteine provided by VanGoudoever et al (1994) was considerably lower (5 mg/g total amino acids) than the concentration that we provided (highNAC = 15 mg/g of total amino acids) in the present experiment and they provided methionine at the total sulfur amino acid requirement in the solution in which N-acetyl-L-cysteine was provided. By supplying methionine at an intake that met the total sulfur requirement, less cysteine may have been required and therefore, more N-acetyl-L-cysteine was excreted. These authors concluded that N-acetyl-L-cysteine was a poor cysteine precursor for parenterally fed premature infants. However, Van Goudoever et al (1994) did not supply the data for urinary N-acetyl-L-cysteine excretion, so we are unable to contrast the urinary excretion of N-acetyl-L-cysteine in their experiment to the present study. However, the nitrogen retention was equal among the treatment groups that Van Goudoever et al (1994) studied, of which the treatment containing N-acetyl-L-cysteine supported similar growth and nitrogen retention to the parenteral formulas that supplied free base L-cysteine and

methionine. Finally, the infants in the VanGoudoever et al (1994) study received lower infusion rates and had lower growth rates than the piglets in the present study and this may have resulted in reduced amino acid utilization. These factors may account for the differences between Van Goudoever et al (1994) and the present study. Other authors have also suggested that the bioavailability of N-acetyl-L-cysteine might be relatively low, as judged by the circulating N-acetyl-L-cysteine concentrations in the venous plasma of adult humans (Borgstrom et al, 1986 and Olsson et al, 1988). However, this is the incorrect manner to assess utilization and would not detect the concentration of N-acetyl-L-cysteine that is rapidly deacylated or taken up by the cells and therefore will underestimate the bioavailability of N-acetyl-L-cysteine.

N-acetyl-L-cysteine has been shown to raise intracellular cysteine and taurine in rat hepatocytes *in vitro* (Banks and Stipanuk, 1994), so although there is rapid clearance of plasma N-acetyl-L-cysteine, there may be an increase in intracellular cysteine, taurine or glutathione. Further to this, Waterfield and Timbrell (1996) demonstrated that urinary taurine increased in a linear fashion to increases in N-acetyl-L-cysteine infusion administered to rats. Post mortem analysis showed that intracellular glutathione and taurine concentrations both increased in response to the N-acetyl-L-cysteine infusion (Waterfield and Timbrell, 1996). Because our study is the first study to measure the bioavailability of N-acetyl-L-cysteine by assessing nitrogen retention, we are the first to demonstrate that in the absence of dietary cysteine and limiting intake of dietary methionine, supplemental N-acetyl-L-cysteine results in increased nitrogen retention and growth. Furthermore, plasma urea concentrations (Figure 3.4.1) were higher in the zeroNAC treatment and remained low and within the reference range in both control and

highNAC treatments. Increased plasma urea is an indication that there is increased amino acid catabolism, which resulted in greater concentrations of plasma urea. The similar interpretation required by the plasma urea and nitrogen retention data in the present study further demonstrate that N-acetyl-L-cysteine was an available precursor of cysteine in neonatal piglets receiving total parenteral nutrition. Therefore, we confidently conclude that N-acetyl-L-cysteine is an effective precursor of cysteine for growth and protein deposition in the neonatal piglet receiving total parenteral nutrition.

Rate of elimination and rate of conversion to cysteine affect the bioavailability of a compound that is infused intravenously. In the present study, the rate of deacylation of N-acetyl-L-cysteine supported equal growth, nitrogen retention and protein deposition as the control, which supplied L-cysteine. N-acetyl-L-cysteine is deacetylated via the enzyme aminoacylase I (EC 3.5.1.14). N-acetyl-tyrosine has been proposed as a precursor for tyrosine; however, N-acetyl-tyrosine is deacylated via aminoacylase III (EC 3.5.1.15) and is not comparable to the deacylation of N-acetyl-cysteine. Aminoacylase I is a cytosolic enzyme (Sjodin et al, 1989) present in the liver, kidney, endothelial tissue, lung and intestinal mucosa and its highest activity occurs in the kidney in the rat (Yamauchi et al, 2002 and Endo, 1980), pig (Henseling et al, 1988), and human (Yamauchi et al, 2002). In the pig, N-acetylmethionine and N-acetyl-L-cysteine are natural substrates for acylase I (Giardina et al, 1997). Acylase I is inhibited by sulfhydryl compounds (Giardina et al, 1997), has a Km of 4.4 mM and requires cobalt and zinc as cofactors (Henseling et al, 1988). When N-acetyl-L-cysteine is added to rat liver, lung and intestine homogenates nearly stoichiometric amounts of L-cysteine were recovered (Sjodin et al, 1989) demonstrating that N-acetyl-L-cysteine is readily

deacetylated to cysteine. Given that acylase I is efficient at deacetylation of N-acetyl-L-cysteine, the deacylation of N-acetyl-L-cysteine to L-cysteine would result in metabolically available L-cysteine. In the present study, plasma N-acetyl-L-cysteine concentrations were low (Figure 3 and 4; ~ 40 μmol/L in lowNAC and ~ 200 μmol/L in highNAC; 1-5% of the infused dose) and plasma cystine showed a trend to increase as the dietary concentration of N-acetyl-L-cysteine increased (Table 3.4.8). However, despite the fact that piglets fed highNAC received an equimolar amount of cysteine as control, the plasma cystine concentrations were higher in the control treatment, which were provided with free L-cysteine (Table 3.4.8, P = 0.09). In addition, plasma N-acetyl-L-cysteine was approximately 2-3 times greater in the highNAC group compared to the lowNAC group (Figure 3.4.3), corresponding to double the amount of N-acetyl-L-cysteine provided to the highNAC group (Table 3.4.1). Although there was a greater amount of N-acetyl-L-cysteine in both the plasma and urine of the highNAC group, the amount of N-acetyl-L-cysteine converted to cysteine was sufficient to support nitrogen retention (Table 3.4.6) and protein deposition equal to the control (Table 3.4.16).

Ahola et al (1999) examined the pharmacokinetics of intravenously delivered N-acetyl-L-cysteine in pre-term infants and found that there was no change in plasma cysteine concentrations and speculated that this may be as a result of N-acetyl-L-cysteine replacing cysteine in mixed disulfides and thus, increasing the availability of free cysteine for cells (Ahola et al, 1999). Ahola et al (1999) reported that plasma N-acetyl-L-cysteine concentrations rose to plateau plasma concentrations within 24 hours of initiation of infusion. This is similar to the present study, where the plasma concentrations in the lowNAC treatment rose within 12 hours and in the highNAC

treatment rose within 48 hours and remained constant for the remainder of the test period (Figure 3.4.4). Clearly, aminoacylase activity was enough to maintain plasma concentrations of N-acetyl-L-cysteine and urinary excretion without allowing an accumulation of N-acetyl-L-cysteine in the plasma. This also suggests that N-acetyl-L-cysteine is an available precursor of cysteine.

Kidney free cystine concentrations of the highNAC paralleled those of the control group (Table 3.4.14); however, cystine concentrations were higher in the control group than all other groups in the jejunum and liver, while we could detect no differences in the longissimus dorsi. Given that the highest activity of Acylase I is found in the kidney (Yamauchi et al, 2002 and Sjodin et al, 1989), increases in kidney free cystine concentrations were associated with increased N-acetyl-L-cysteine intake. Equal kidney free cystine concentrations in the highNAC and control group indicate that cystine is being released in equal concentrations from N-acetyl-L-cysteine or glutathione. These similarities in kidney cystine concentrations between highNAC and control further suggest that N-acetyl-L-cysteine is an effective precursor to cysteine.

The control and highNAC groups had similar plasma methionine concentrations, which were higher than the zeroNAC and lowNAC groups. This may occur via less methionine being transsulfurated to produce cysteine in these groups. This suggests that the availability of cysteine (from L-cysteine or N-acetyl-L-cysteine) was similar and did not necessitate increased transsulfuration of methionine to cysteine when N-acetyl-L-cysteine was provided in the diet.

Liver (Table 3.4.12), jejunal (Table 3.4.13) and kidney (Table 3.4.14) taurine concentrations were consistently higher in the control group than all other groups.

Although tissue free amino acid concentrations cannot explain individual differences in how each amino acid is handled on a cellular or tissue basis, free amino acid concentrations can give insight into the net effect of dietary input, protein turnover, biosynthesis of amino acids, transamination reactions, transport rates across tissue and organelle membranes and oxidation rates (Bertolo et al, 2000). Using rat hepatocytes, Banks and Stipanuk (1994) demonstrated that N-acetyl-L-cysteine was utilized less than cysteine for sulfate and taurine production but a higher percentage of N-acetyl-L-cysteine (62-81% vs. 46%, respectively) was used for glutathione synthesis compared to cysteine. These researchers speculated that the lower rate of utilization of N-acetyl-L-cysteine for taurine and sulfate production could be partly explained by a lower rate of substrate availability, but did not speculate what the mechanism that favoured the conversion of N-acetyl-L-cysteine to glutathione may be. The present data agrees with the data of Banks and Stipanuk (1994) in that L-cysteine produced more taurine than an equimolar amount of N-acetyl-L-cysteine. We speculate that N-acetyl-L-cysteine may promote glutathione synthesis and it is glutathione that then acts as a reserve pool of cysteine for protein synthesis. Further research should focus on the difference in metabolic handling between L-cysteine and N-acetyl-L-cysteine.

Although N-acetyl-L-cysteine and L-cysteine (highNAC and control, respectively) supplementation of a complete total parenteral formula resulted in some significant differences in amino acid metabolism (as evidenced by the differences in tissue and plasma amino acid concentrations) these diets supported similar plasma urea, nitrogen retention and growth.

3.4.5 **Conclusions**

These data clearly indicate that we must reject our null hypothesis that N-acetyl-L-cysteine is not an available precursor of cysteine. We conclude that N-acetyl-L-cysteine is a highly bioavailable (~ 85%) precursor for L-cysteine for nitrogen retention, protein deposition and growth in neonatal piglets receiving total parenteral nutrition. This evidence contradicts studies that showed that N-acetyl-L-cysteine was a poor precursor to cysteine as measured by a high excretion rate of N-acetyl-L-cysteine (Van Goudoever et al, 1994). However, these studies did not measure the critical biological outcomes of nitrogen retention and growth. Our study clearly demonstrates that in the neonatal piglet, N-acetyl-L-cysteine can support similar rates of nitrogen retention and growth as an equimolar intake of L-cysteine. The clinical concerns regarding high methionine intakes in parenteral solutions, and the abnormally low plasma cysteine concentrations observed in parenterally fed neonates, indicate that cysteine supplementation to total parenteral nutrition must be considered. We conclude that N-acetyl-L-cysteine offers an efficacious method for supplying L-cysteine during parenteral feeding.

3.4.6 **Literature cited**

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3.4.7 APPENDIX

Table 3.4.17: N-acetylcysteine retention (%) of piglets receiving different dietary concentrations of N-acetylcysteine or a control diet.

Day	zeroNAC	control	lowNAC	highNAC	pooled SE
3[1]	0¥	0¥	91.07*	87.72*	2.25
4	0	0	90.59	85.32	2.02
5	0	0	85.77	85.04	2.73
6	0	0	80.10	80.62	2.80
7	0	0	79.40	74.36	2.75
Mean	0	0	85.39	82.61	1.73

[1]Values are means of N=5 piglets. Values are expressed as N-acetylcysteine retention (%)= (NAC intake – NAC output)/nitrogen intake x 100%.
¥ Urine was analyzed but NAC was below the level of detection.
*Data was analyzed using repeated measures ANOVA, is P value <0.05 for the comparison then the shape of the curve was defined. Both lowNAC and highNAC showed a significant linear decline.

Table 3.4.18: Urinary tyrosine (μmol/L)[1] of piglets receiving different dietary concentrations of N-acetylcysteine or a control diet.

Amino acid (μmol/L)	Control	zeroNAC	lowNAC	highNAC	pooled SE
Day 3[2]	4^{b}	50^{a}	25^{ab}	44^{ab}	6
Day 4	4^{b}	69^{a}	37^{ab}	49^{ab}	9
Day 5	10	59	30	32	8
Day 6[3]	8	46	34	35	6
Day 7[3]	9	49	56	21	8

[1]Values are means of N=5 piglets.
[2]Overall ANOVA, F-test, P<0.01. For data in a row with different superscript letters indicate a significant difference between diet treatments (P<0.05); all others are not different.
[3]Urine concentrations of tyrosine tended (P<0.13) tended to be greater in zeroNAC and lowNAC than all other treatment groups.

Table 3.4.19: Urinary phenylalanine (μmol/L)[1] of piglets receiving different dietary concentrations of N-acetylcysteine or a control diet.

Amino acid (μmol/L)	Control	zeroNAC	lowNAC	highNAC	pooled SE
Day 3	10	61	20	16	11
Day 4	9	22	16	17	3
Day 5	11	29	20	19	4
Day 6	9	20	18	17	3
Day 7	7	55	29	10	10

[1]Values are means of N=5 piglets.

Table 3.4.20: Urinary lysine (μmol/L)[1] of piglets receiving different dietary concentrations of N-acetylcysteine or a control diet.

Amino acid (μmol/L)	Control	zeroNAC	lowNAC	highNAC	pooled SE
Day 3[2]	186[ab]	335[a]	103[b]	67[b]	35
Day 4	213	281	229	123	35
Day 5	281	283	128	131	46
Day 6	303	302	150	129	42
Day 7	271	233	232	113	36

[1]Values are means of N=5 piglets.
[2]Overall ANOVA, F-test, $P<0.01$. For data in a row with different superscript letters indicate a significant difference between diet treatments ($P<0.05$); all others are not different.

3.5 THE REGULATION OF SULFUR AMINO ACID METABOLISM IN RESPONSE TO CYSTEINE INTAKE IN NEONATAL PIGLETS RECEIVING PARENTERAL OR ENTERAL NUTRITION.

3.5.1 Introduction

The National Research Councils' (1998) Nutrient Requirements for Swine recommends that methionine and cysteine be supplied in a 50: 50 ratio, cumulatively supplying 0.5 g kg^{-1} d^{-1} of sulfur amino acids for pigs weighing between 1-5 kg. We carried out a series of indicator amino acid oxidation studies and determined a mean total sulfur amino acid requirement of 0.42 and 0.29 g kg^{-1} d^{-1} for enterally and parenterally fed piglets, respectively (Shoveller et al, 2003a; Chapter 3.1). Subsequently, we estimated the requirement for methionine in the presence of excess cysteine (0.5 g kg^{-1} d^{-1}) and found a mean methionine requirement of 0.25 and 0.18 g kg^{-1} d^{-1} for enteral and parenteral feeding respectively (Shoveller et al., 2003b; Chapter 3.2). In the enterally fed situation, the total sulfur amino acid requirement (0.42 g kg^{-1} d^{-1}) and the capacity of cysteine to spare the methionine requirement (40% of the methionine requirement), closely compare to the recommendation of the NRC (1998) and with previous estimates of the sulfur amino acid requirement. We defined, for the first time, the total sulfur amino acid requirement and cysteine sparing capacity during parenteral feeding in the neonatal piglet. Although we demonstrated that addition of dietary cysteine did not improve overall protein synthesis, we did find that the parenterally fed piglets receiving a cysteine free diet had the lowest plasma total cysteine concentrations compared to all other treatment groups (Shoveller et al., 2003b; Chapter 3.2). Therefore, either cysteine

synthesis is limited (but not restricted) or cysteine use for glutathione, taurine or sulfate synthesis is greater in the parenterally fed, compared to the enterally fed, neonatal piglet.

These results led us to speculate that there was either a secondary use for cysteine or lower cysteine synthesis during parenteral feeding as compared to enteral feeding; therefore, cysteine synthesis (as defined by methionine oxidation, **Figure 3.5.1**) may be lower and/or cysteine oxidation (**Figure 3.5.2**) may be higher during parenteral vs. enteral feeding. If there is a secondary use for cysteine in parenterally fed piglets and there is not a limitation in the transsulfuration pathway, then cysteine synthesis (Figure 3.5.1) must be higher in parenterally fed piglets. In support of this hypothesis, Mosharov et al (2000) demonstrated that oxidant stress, such as that observed in neonates (Shalhal et al, 1991), increases transsulfuration *in vitro*. Methionine is metabolized through transmethylation to homocysteine. Homocysteine then condenses with serine, in an irreversible reaction, to produce cystathionine. Cystathionine is then hydrolyzed to α-ketobutyrate and cysteine, and the oxidation of α-ketobutyrate releases the 1 carbon of methionine. Therefore, measurement of methionine oxidation provides a measurement of cysteine synthesis or transsulfuration. By measuring transsulfuration in both parenterally and enterally fed piglets at the same dietary concentrations of the sulfur amino acids, we will be able to determine if transsulfuration is different between routes of feeding. Furthermore, we will also measure cysteine oxidation (Figure 3.5.2), at the same dietary cysteine concentrations, to determine whether an increase in cysteine oxidation during parenteral feeding can account for the low plasma cysteine concentrations observed during parenteral feeding.

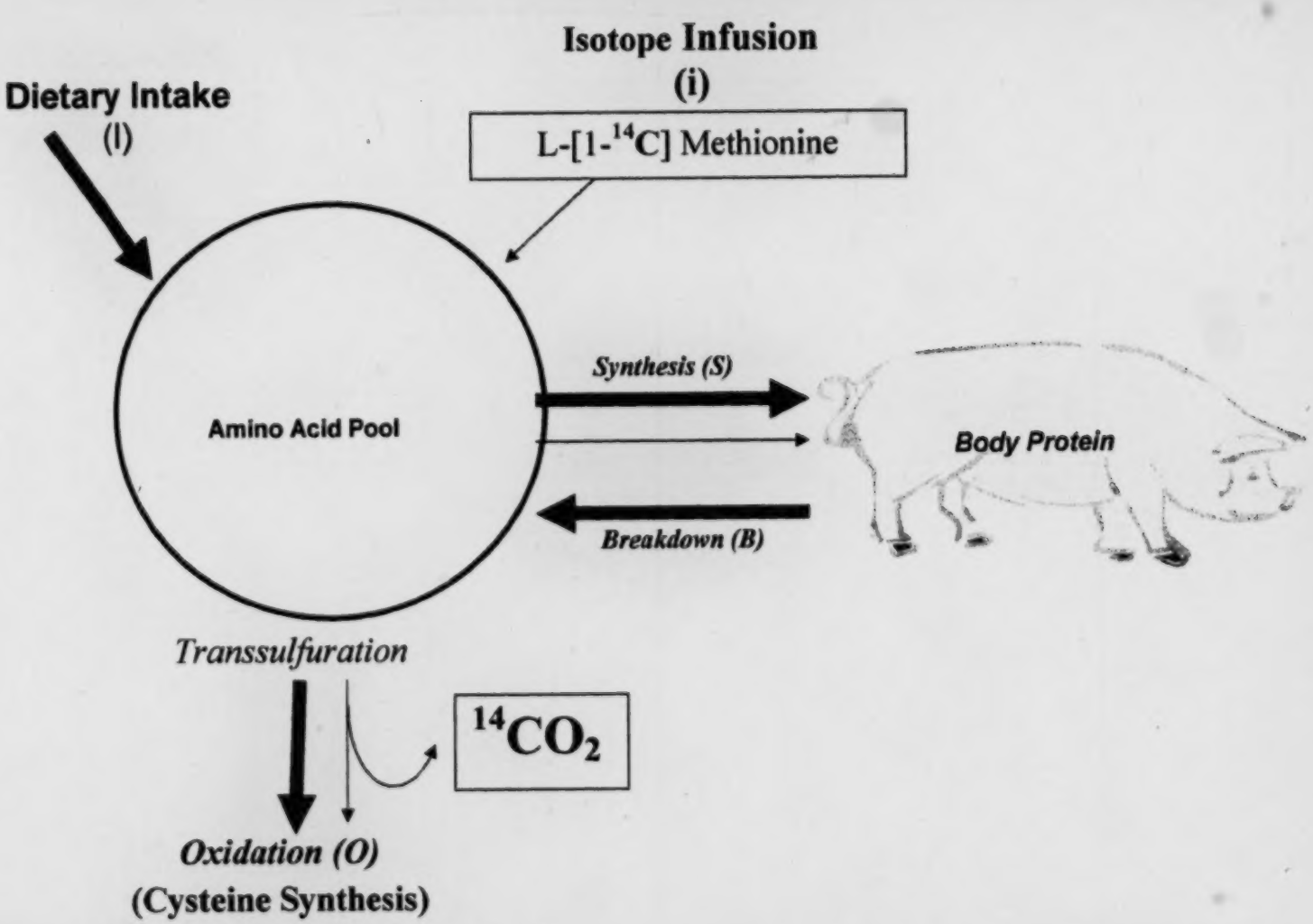


Figure 3.5.1: Model of methionine metabolism. Terminal output of methionine can only occur via oxidation through the transsulfuration pathway.

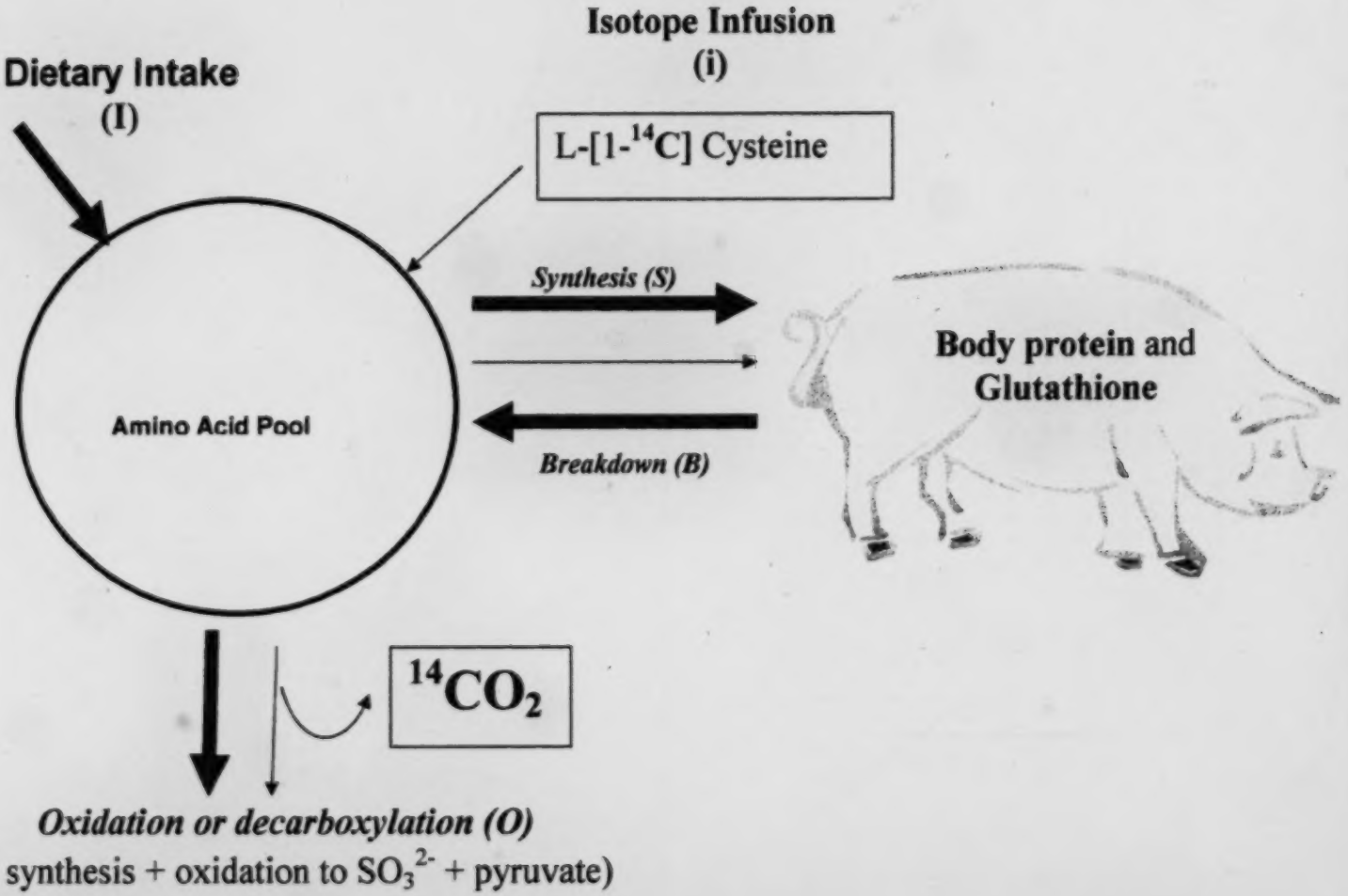


Figure 3.5.2: Model of cysteine metabolism. Terminal output of cysteine can only occur via decarboxylation to taurine or oxidation to pyruvate and SO_3^-

A series of experiments examining the response of methionine kinetics to varying intakes of methionine and cysteine suggested that there was no cysteine sparing mechanism in humans (Storch et al, 1990, Hiramatsu et al, 1994, Raguso et al, 1997, Fukagawa et al, 1998, Raguso et al, 1999, Raguso et al, 2000). However, these studies provided the sulfur amino acids at a level consistent with the 1985 FAO/WHO/UNU, which DiBuono et al (2001a and 2001b) have identified as being inadequate. Finkelstein and Mudd (1967) demonstrated that feeding cysteine resulted in the reduction of cystathionine-β-synthase (CBS, EC 4.2.1.22) activity, providing a mechanism by which cysteine exerts its sparing effect upon the methionine requirement. Others have used growth, feed efficiency and/or nitrogen balance to demonstrate that cysteine can replace part of the methionine requirement (Shelton et al, 1951, Curtin et al, 1952, Becker et al, 1955, Mitchell et al, 1968, Baker et al, 1969, Kim and Bayley, 1983, Roth et al, 1989). In order to demonstrate a response in cysteine synthesis to increasing dietary cysteine, we have provided dietary methionine at 50% of the recommended total sulfur amino acid requirement (NRC, 1998), which is sufficient to meet the mean methionine obligatory requirement. If dietary cysteine reduces cysteine synthesis via a reduction in transsulfuration then transsulfuration (as represented by methionine oxidation) will be reduced as dietary cysteine is increased.

Thus, the present study was designed to directly determine whether increasing cysteine intake, when methionine intake is held constant, results in a change in transsulfuration (as measured by methionine oxidation) or cysteine oxidation. These data will also provide insight into how the gut affects whole body cysteine balance, because

of simultaneous measures of transsulfuration or cysteine synthesis (inputs) and oxidation (losses).

3.5.2 **Materials and Methods**

3.5.2.1 ***Piglets and study protocol.***

The Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee at the University of Alberta approved all procedures in this study. A total of 64 male Landrace/Large White intact piglets (Genex Swine Group) were obtained from the University of Alberta, Swine Research and Technology Centre (Edmonton, AB, CAN). The piglets were weighed and then pre-anaesthetized with acepromazine (0.5 mg/kg; Atravet™; Ayerst Laboratories, Montreal, PQ) and maintained during surgery with 3-4% isoflourane. All piglets that were allocated to the intravenous treatment (n=32) were then fitted with two venous catheters (Ed-Art, Don Mills, Canada) using the modified methods of Wykes et al. (1993). Enterally fed piglets (n=32) had a venous catheter implanted (femoral) and gastric catheters were inserted using the method of Rombeau et al. (1984). In intravenously fed pigs, an infusion catheter was inserted into the left jugular vein and advanced to the superior vena cava just cranial to the heart. In both intravenously and enterally fed piglets, a sampling catheter was inserted into the left femoral vein and advanced to the inferior vena cava just caudal to the heart. After surgery, incision sites were treated with a topical antibiotic (Hibitane Veterinary Ointment: Ayerst Laboratories, Montreal, PQ) and an analgesic (0.1 mg/kg Buprenex, Buprenorphrine HCl, Reckitt and Colman Pharmaceutical Inc., Richmond, VA) was given intramuscularly immediately and again 8h-post surgery. Piglets were then put into

cotton jackets, which secured the tether to the piglets. The tether was part of the swivel-tether system (Alice King Chatham Medical Arts, Los Angeles, California), that enabled the pig to move freely while receiving a continuous dietary infusion, ensuring that the catheters did not become tangled or occluded.

3.5.2.2 ***Animal housing.***

Piglets were housed in individual circular cages, 75 cm in diameter and toys were added to enhance their environment. The animal rooms were maintained at an ambient temperature of 25°C, with supplemental heat supplied by heat lamps. The lighting schedule was 12 h of light commencing at 0600h.

3.5.2.3 ***Diet regimen.***

Elemental diets were provided as continuous infusions by pressure sensitive infusion pumps. Piglets received 15 g amino acids $kg^{-1} \cdot d^{-1}$ and 1.1 MJ metabolizable energy $kg^{-1} \cdot d^{-1}$ with glucose and lipid (Intralipid 20%, Fresenius-Kabi, Stockholm, Sweden) each supplying 50% of nonprotein energy intake. The base amino acid profile of the complete elemental diet fed during adaptation (d 0 until d 5) has been previously described (Shoveller et al., 2003a, Chapter 3.1). The amino acid profile was based on human milk protein (Vaminolact: Fresenius-Kabi, Stockholm, Sweden) except phenylalanine and tyrosine which were provided at their estimated safe levels of intake (House et al., 1997a, and 1997b) and arginine was provided at 1.2 $g \cdot kg^{-1} \cdot d^{-1}$ (Brunton et al., 1999). Diet infusion rates were adjusted daily after weighing the piglets. Vitamins were supplied as a commercial solution, MVI Paediatric (Rhone-Poulenc Rorer Canada Inc, Montreal, PQ) which was added to the diet immediately prior to feeding. The cofactors involved in the transsulfuration pathway, vitamin B-12, choline, B-6 and folate

were provided via the MVI solution at approximately 115% of requirement (NRC, 1998). Piglets also received a mineral solution including zinc, copper, manganese, chromium, selenium and iodide at 200% of the NRC (1998) recommendation for piglets. Iron was supplied as iron dextran solution in the TPN (16 mg/mL).

TPN was initiated immediately following surgery, and increased to full infusion rates (13.5 ml· kg^{-1} ·h^{-1}) by the end of day 1 (Shoveller et al., 2003a; Chapter 3.1). Of the 64 piglets, 32 were allocated to each of the intravenously and enterally fed groups. All piglets received both diet and isotope by their allocated route of feeding. Complete TPN was continued until 1800 on d 5. Piglets were then randomly allocated to one of the eight test levels of cysteine (0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.40, 0.50 g kg^{-1} d^{-1}) with a constant concentration of methionine (0.25 g kg^{-1} d^{-1}). Cysteine was provided as L-cysteine free base in all test diets. All test diet solutions were made isonitrogenous by altering the concentration of L-alanine. The solutions were sterilized with a 0.22-μm filter (Millipore, Milford, MA). Due to the unstable nature of L-cysteine in aqueous solutions, test diets were made immediately prior to infusions. Piglets were maintained on a test diet from 1800h on d 5 until the completion of the second oxidation study and subsequent necropsy on d 8.

3.5.2.4 ***Tracer infusion, $^{14}CO_2$ collection and analytical procedures.***

Methionine and cysteine infusion experiments were conducted on d 6 and d 8 in a cross-over design resulting in an equal number of piglets receiving methionine and cysteine isotope on d 6 and d 8. Methionine oxidation represents cysteine synthesis, or the rate of transsulfuration, as the release of 1 carbon of methionine can only occur via the transsulfuration pathway. Methionine and cysteine oxidation were determined by a

primed (186 kBq (5 μCi/kg)), constant intravenous infusion (186 kBq (5 $\mu Ci \cdot kg^{-1} \cdot h^{-1}$)) of a tracer solution containing 92.8 MBq (2.5 mCi)/L of L-[1-^{14}C]methionine or L-[1-^{14}C]cysteine (200 MBq (54 mCi/mmol; American Radiolabeled Chemicals, Inc. St. Louis, MO). The constant infusion was 6h, in order to achieve plateau in breath labeling. One hour of background $^{14}CO_2$ collection was taken on d 8 and background was subtracted from total $^{14}CO_2$ collected during the infusion. Details of infusion protocol, $^{14}CO_2$ collection and blood collection procedures have been described previously (House, 1997a). Following the infusion on d 8, piglets were anaesthetized with isoflourane and killed by injection of 1000 mg of sodium pentobarbital into a venous catheter.

3.5.2.5 ***Calculations.***

The rate of $^{14}CO_2$ expiry (dpm kg^{-1} h^{-1}) was determined and data were corrected for the retention of label in the bicarbonate pool using a bicarbonate retention factor (BRF) of 0.933 (Wykes et al. 1993). The resulting equations appear as follows:

Corrected $V^{14}CO_2$ (dpm kg^{-1} h^{-1})

= $V^{14}CO_2$ (dpm kg^{-1} h^{-1}) /BRF

Percent of dose oxidized

= plateau corrected $V^{14}CO_2$ / isotope infusion (dpm kg^{-1} h^{-1})

3.5.2.6 ***Statistical analyses.***

A completely randomized design, with cysteine intake serving as the main treatment effect, was used in this experiment. Significant differences in cysteine synthesis and oxidation among cysteine intakes were determined using an ANOVA. If P values were <0.05 for the F-value of the ANOVA model, significant differences among

treatments were determined using the Student Newman Keul's multiple comparison procedure (SAS/STAT, version 8.1, SAS Institute, Cary, NC).

Determination of the dietary intake of cysteine required to reduce cysteine synthesis (methionine oxidation) to obligatory methionine oxidation, was performed using a two-way linear crossover model, as described previously (Seber, 1977, Ball and Bayley, 1984). Regression analysis variables were dietary concentration of cysteine as the independent variable and percentage of methionine dose oxidized as the dependent variable. To determine the amount of dietary cysteine required to reduce methionine oxidation to obligatory methionine oxidation levels in both parenteral and enteral treatments, the data points were partitioned between two distinct regression lines. The final regression was chosen as the model that produced the highest regression coefficients for the dependent variables. The point at which the two regression lines intersected provides an estimate of the maximum amount of cysteine sparing. The 95% confidence intervals, for the estimation of a safe level of intake, were also determined.

The effects of cysteine intake on cysteine oxidation were analyzed using PROC REG (SAS/STAT, version 8.1, SAS Institute, Cary, NC) and if an effect was defined, we considered the 95% asymptote as the minimal cysteine oxidation.

To compare the breakpoints in cysteine synthesis (methionine oxidation), the breakpoint was treated as a sample mean and the pooled two-sample t procedure was applied (Pagano and Gavreau, 2000). Based on the assumption that the subjects were derived from the same population and identical procedures were used, the true variance was assumed to be the same. Calculation of pooled variance was used to adjust each sample variance with weighting equal to its degrees of freedom. Therefore, pooled

variance for the two groups was determined and used to test if the two breakpoints were different using a pooled two-sample t-procedure.

Differences between routes of feeding in the plateau region of cysteine synthesis, representing obligatory methionine oxidation, were compared using a two-tailed unpaired t-test (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). Differences between routes of feeding in cysteine oxidation were compared using a two-tailed paired t-test (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

3.5.3 **Results**

3.5.3.1 ***Piglet performance.***

All piglets were healthy during the course of this study. For enterally fed piglets, piglet weight upon arrival (1554 g ± 27) and weight at study (2539 g ± 6) did not differ among dietary cysteine levels ($P>0.05$). In addition, rates of average daily gain for the 5-day test period before test diet initiation did not differ (162 g/d ± 6) ($P>0.05$). For parenterally fed piglets, piglet weight upon arrival (1640 g ± 30 (pooled SE)) and weight at study did (2527 g ± 48) did not differ among dietary cysteine levels ($P>0.05$). In addition, rates of average daily gain for the 5-day period before test diet initiation also did not differ (143 g/d ± 4) ($P>0.05$).

3.5.3.2***Enteral feeding/isotope study.***

Values for $^{14}CO_2$ recovery for both methionine and cysteine infusion are summarized in **Table 3.5.1**. Plateaus in breath $^{14}CO_2$ from 1-^{14}C- methionine were

Table 3.5.1: $^{14}CO_2$ from L-[1-^{14}C]methionine and L-[1-^{14}C]cysteine in piglets receiving total enteral nutrition with graded levels of dietary cysteine and 0.25 g kg^{-1} d^{-1} methionine[1]

	Cysteine Intake (g·kg^{-1}· d^{-1})									
	0	0.05	0.1	0.15	0.2	0.25	0.4	0.5	pooled	ANOVA
n	4	4	4	4	4	4	4	4	SE	P value
percent of methionine oxidized[2]	20.6^{a}	16.0 ^{ab}	11.6 ^{bc}	7.0 ^{cd}	6.1^{cd}	6.0^{cd}	5.1^{cd}	3.4^{d}	1.15	<0.0001
percent of cysteine oxidized	22.8	17.4	14.2	13.5	17.0	17.2	17.3	18.0	0.86	NS[3]

[1] Values represent the means of 4 pigs per dietary cysteine intake. Values represent the percent of dose oxidized at isotopic steady state.
[2]Overall ANOVA, F-test, P<0.05. Values with different superscript letters indicate a significant difference among diet levels (Student Newman Keul's multiple comparisons procedure).
[3] Non-significant (P>0.05).

reached within 4 hours after the initiation of the primed constant infusion in all pigs. Plateaus in breath $^{14}CO_2$ from 1-^{14}C-cysteine were reached within 1 hour after the initiation of the primed constant infusion. Transsulfuration (methionine oxidation), expressed as a percentage of the methionine dose oxidized during isotopic steady state, was significantly influenced by cysteine intake ($P<0.0001$, **Figure 3.5.3**). As cysteine intake increased from 0 to 0.15 g kg^{-1} d^{-1}, transsulfuration decreased (slope= -90 % dose oxidized/ 100 mg of cysteine intake, $P=0.0004$). Further increases in cysteine intake (from 0.15 to 0.5 g cysteine/ kg BW d^{-1}) resulted in a decrease in transsulfuration (slope = -8 % dose oxidized/ 100 mg of cysteine intake, $P=0.005$). The slope of the second line was different from zero; therefore, increasing cysteine intake resulted in a reduction in transsulfuration, but at a slower rate of change than the first regression line. The breakpoint estimate for transsulfuration or cysteine synthesis (Figure 3.5.3) was 0.15 g kg^{-1} d^{-1} (95% confidence interval: 0.11-0.20 g kg^{-1} d^{-1}).

There were no significant differences in cysteine oxidation among dietary cysteine intakes during enteral feeding ($P>0.05$, **Figure 3.5.4**). When the data were regressed against cysteine intake, there was a significant polynomial relationship ($P = 0.0006$, $R^2 = 0.37$, MSE = 3.1) between cysteine oxidation and increasing cysteine intake. Cysteine oxidation was minimized at 0.25 g kg^{-1} d^{-1}dietary cysteine intake.

3.5.3.3 ***Parenteral feeding/isotope study.***

Values for $^{14}CO_2$ recovery for both methionine and cysteine infusion are summarized in **Table 3.5.2**. Plateaus in breath $^{14}CO_2$ from 1-^{14}C- methionine were reached within 4.5 hours after initiation of the primed constant infusion in all pigs.

Figure 3.5.3 : Cysteine synthesis, defined by the oxidation of L-[1-^{14}C]methionine as a percentage of dose, in enterally fed piglets receiving graded levels of cysteine and 0.25 g kg^{-1} d^{-1} methionine (n=32). The break-point value was 0.15 g kg^{-1} d^{-1} with a confidence interval of 0.11-0.20 g kg^{-1} d^{-1}.

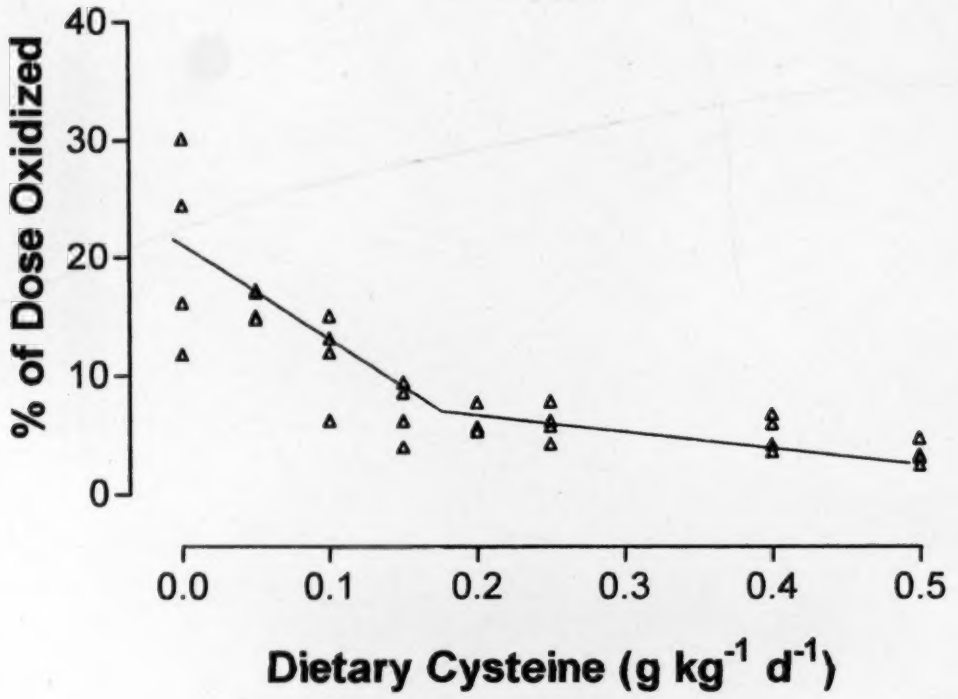


Figure 3.5.4 : Oxidation of L-[1-^{14}C]cysteine as a percentage of dose in enterally fed piglets receiving graded levels of cysteine and 0.25 g kg^{-1} d^{-1} methionine (n=32). Cysteine oxidation was associated with cysteine intake in a second order polynomial response (y= 20.09 + 27.79x + 71.70x^2; P<0.05, R^2=0.12) with cysteine oxidation minimized at 0.25 g cysteine kg^{-1} d^{-1}.

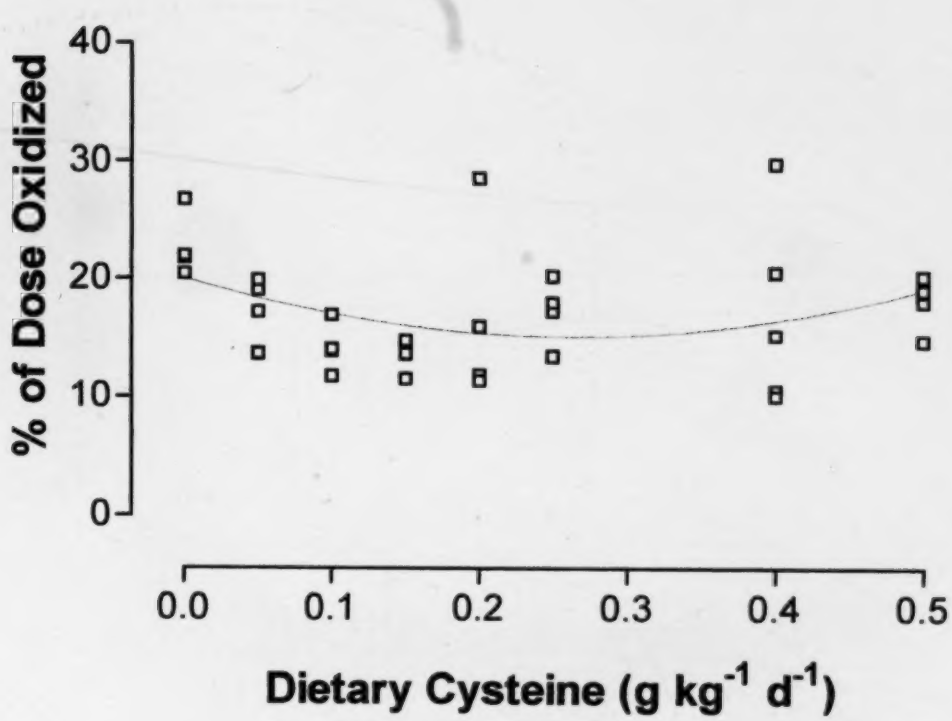


Table 3.5.2 : $^{14}CO_2$ from L-[1-^{14}C]methionine and L-[1-^{14}C]cysteine in piglets receiving total parenteral nutrition with graded levels of dietary cysteine and 0.25 g kg^{-1} d^{-1} methionine[1]

	Cysteine Intake ($g \cdot kg^{-1} \cdot d^{-1}$)									
	0	0.05	0.1	0.15	0.2	0.25	0.4	0.5	pooled	ANOVA
n	4	4	4	4	4	4	4	4	SE	P value
percent of methionine oxidized[2]	22.5[a]	17.8[b]	15.7[b]	10.6[c]	8.4[c]	8.3[c]	7.9[c]	3.8[d]	1.10	<0.0001
percent of cysteine oxidized	21.5	17.0	19.8	21.3	20.2	19.4	13.9	23.7	1.06	NS[3]

[1] Values represent the means of 4 pigs per dietary cysteine intake. Values represent the percent of dose oxidized at isotopic steady state.
[2]Overall ANOVA, F-test, $P<0.05$. Values with different superscript letters indicate a significant difference among diet levels (Student Newman Keul's multiple comparisons procedure).
[3] Non-significant ($P>0.05$).

Plateaus in breath $^{14}CO_2$ from 1-^{14}C-cysteine were reached within 1 hour after the initiation of the primed constant infusion in all pigs. Transsulfuration, expressed as a percentage of the methionine dose oxidized, was significantly influenced by cysteine intake ($P<0.0001$, **Figure 3.5.5**). As cysteine intake increased from 0 to 0.20 g kg^{-1} d^{-1}, methionine oxidation decreased (slope = -71 % dose oxidized/ 100 mg of cysteine intake, $P<0.0001$). Further increases in cysteine intake, up to and including 0.40 g kg^{-1} d^{-1}, did not affect cysteine synthesis ($P>0.05$). However, at the highest intake, 0.5 g kg^{-1} d^{-1}, cysteine synthesis was significantly lower ($P<0.05$) than all other intakes; these data were not included in the dual regression breakpoint model and will be discussed separately. The breakpoint estimate for transsulfuration or cysteine synthesis (Figure 3.5.5) was 0.17 g kg^{-1} d^{-1} (95% confidence interval: 0.12-0.23 g kg^{-1} d^{-1}).

Cysteine oxidation was not different among levels of cysteine intake ($P>0.05$, **Figure 3.5.6**). Of note, is the increased variation associated with the cysteine oxidation data as dietary cysteine intake was increased.

3.5.3.4 ***Comparison of the enteral and parenteral breakpoint estimate for transsulfuration.***

There was no difference ($P>0.05$) between the breakpoint for transsulfuration or the maximal cysteine sparing capacity, as defined by the breakpoint in methionine oxidation, for parenterally vs. enterally fed piglets ($P>0.05$). These data indicate that the absolute intake of cysteine required to reduce transsulfuration to basal levels was similar between parenteral and enteral routes of feeding.

Figure 3.5.5 : Cysteine synthesis, defined by the oxidation of L-[1-^{14}C]methionine as a percentage of dose, in parenterally fed piglets receiving graded levels of cysteine and 0.25 g kg^{-1} d^{-1} methionine (n=32). The break-point value was 0.17 g kg^{-1} d^{-1} with a confidence interval of 0.12-0.23 g kg^{-1} d^{-1}.

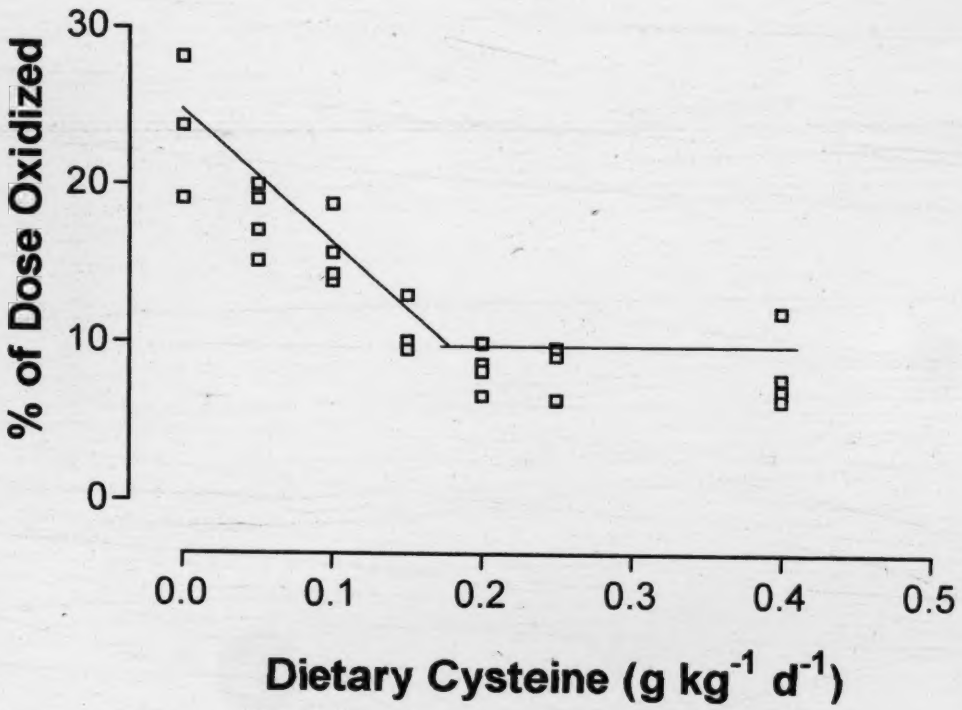
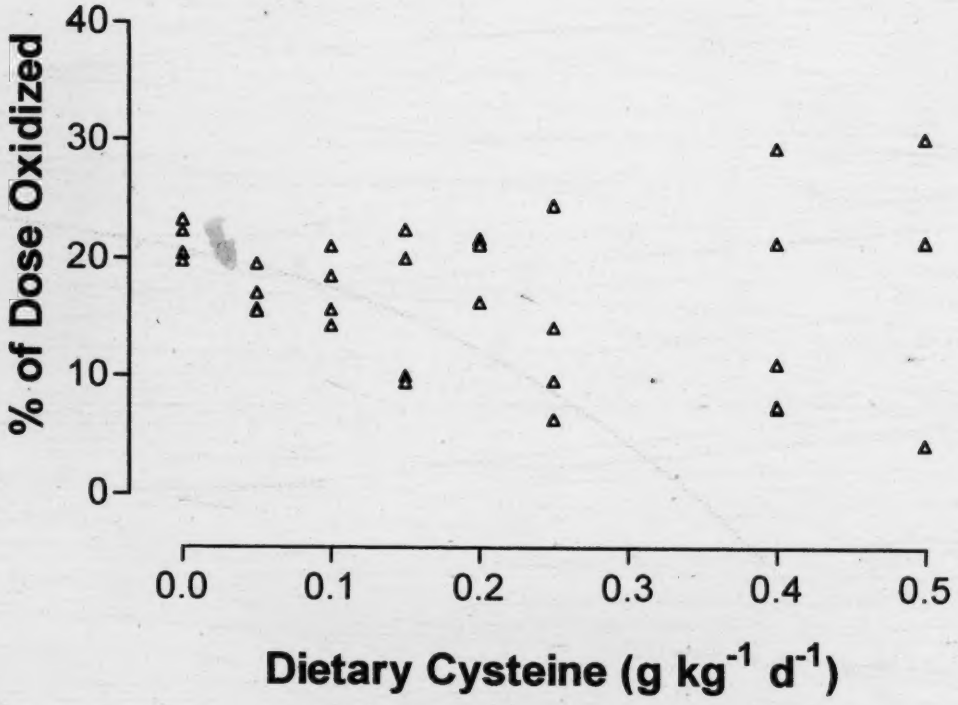


Figure 3.5.6: Oxidation of L-[1-^{14}C]cysteine as a percentage of dose in parenterally fed piglets receiving graded levels of cysteine and 0.25 g kg^{-1} d^{-1} methionine (n=32). Cysteine oxidation was not significantly affected by dietary cysteine.



3.5.3.5 ***Obligatory methionine oxidation in enterally and parenterally fed piglets.***

After the breakpoint was achieved for methionine oxidation, the remaining dietary intakes of cysteine were assumed to represent an estimate of the obligatory methionine oxidation. The mean methionine oxidation during enteral feeding (n=16) was 5.16 ± 1.58 (SD) and during parenteral feeding (n=12) was 8.07 ± 2.02. Obligatory methionine oxidation was significantly higher in parenteral feeding as compared to enteral feeding (P=0.0008).

3.4.3.6 ***Comparison of delay in $^{14}CO_2$ recovery.***

In both routes of feeding, a plateau was achieved in breath $^{14}CO_2$ at approximately 4 hours after initation of the primed-constant infusion of 1-^{14}C-methionine. In contrast, with 1-^{14}C-cysteine the plateau was achieved in breath $^{14}CO_2$ within 1 hour of initiation of primed-constant infusion. This suggests that there was a delay in the release of the carboxy carbon of methionine compared to cysteine, likely due to the additional metabolic functions of methionine. Furthermore, this means that we underestimated the prime for methionine; however, this equimolar infusion of methionine and cysteine allowed detection of this delay in methionine oxidation.

3.5.4 **Discussion**

Cysteine is synthesized from the sulfur moiety of methionine and from the indispensable amino acid serine; if methionine is provided in quantities large enough to meet the total sulfur amino acid requirement, then cysteine is fully dispensable from the diet. To our knowledge, this is the first *in vivo* examination of the effects of increasing

dietary cysteine, with a constant methionine supply, on both methionine and cysteine oxidation. This study clearly demonstrated that as cysteine intake increased, transsulfuration, as measured by methionine oxidation, decreased in a linear fashion until the dietary requirement for cysteine was met or exceeded (Figures 3.5.3 and 3.5.5), in both enterally and parenterally fed piglets, respectively. In enterally fed piglets, transsulfuration decreased until the sum of dietary methionine (0.25 g kg^{-1} d^{-1}) and cysteine (0.15 g kg^{-1} d^{-1}) equaled 0.40 g kg^{-1} d^{-1} total sulfur amino acid intake. This decrease in transsulfuration with increasing cysteine intake between 0 and 0.15 g kg^{-1} d^{-1} represents cysteine sparing the methionine requirement for protein synthesis. The present estimate of the total sulfur amino acid requirement, using minimum transsulfuration, is similar to our previous estimate of the mean total sulfur amino acid requirement (0.42 g kg^{-1} d^{-1}) determined by indicator amino acid oxidation in neonatal piglets receiving an enteral diet providing methionine only (Shoveller et al., 2003a; Chapter 3.1). If the present estimate of 0.40 g kg^{-1} d^{-1} is corrected on a molar basis, it represents 0.44 g kg^{-1} d^{-1} methionine equivalents. These data also agree with previous data demonstrating that methionine oxidation was reduced when cysteine partially replaced dietary methionine in adult humans receiving an oral diet (DiBuono et al., 2004).

Increases in cysteine intake in enterally fed piglets, from 0.15 to 0.50 g kg^{-1} d^{-1}, resulted in a small, but significant, reduction in transsulfuration; there are several possible explanations for this response that must be considered. This reduction may be due to a distribution among piglets in the total sulfur amino acid requirement, resulting in detection of a small sparing effect beyond the population mean requirement for the total

sulfur amino acids. However, the 95% confidence interval (0.11-0.20 g cysteine kg^{-1} d^{-1}) for the mean requirement (0.15 g cysteine kg^{-1} d^{-1}) suggests that either the population distribution of the methionine requirement is skewed dramatically to the right (i.e. is greater than 3 times the mean requirement), which is unlikely, or that some other explanation is required. Alternatively, the second regression line may represent the effect of excess cysteine on the regulation of the transsulfuration enzyme CBS. Other researchers have shown that rats fed a cysteine supplemented diet had lower *in vitro* hepatic CBS activity compared to rats fed a diet containing methionine alone (Finkelstein and Mudd, 1967; Stipanuk and Benevenga, 1977). The number of cysteine intakes (n=4) and corresponding data points (n=16) above the total sulfur amino acid requirement may have made the present experiment sensitive enough to detect this small effect on the second regression line (Figure 3.5.3). Many other experiments have examined dietary cysteine intakes at or above the requirement, which would fall on the second, less pronounced, regression line (Storch et al, 1990, Hiramatsu et al, 1994, Raguso et al, 1997, Fukagawa et al, 1998, Raguso et al, 1999 and Raguso et al, 2000). Because the response to dietary cysteine intakes above the total sulfur amino acid requirement is very small, experiments using fewer diets and subjects may not detect significant differences between dietary cysteine intakes.

These data also demonstrate that when the total sulfur amino acid intake exceeded the requirement, using cysteine supplementation, ~5-8 % of dietary methionine intake was oxidized in both enterally and parenterally fed piglets. Although the present study cannot identify whether the obligatory oxidation of methionine was via transsulfuration or transamination (Mitchell and Benevenga, 1978), it is likely via transsulfuration

because the presence and significance of the transamination pathway has not been demonstrated in pigs or humans. These data also mean that even when dietary cysteine intake is in excess, the rate of transsulfuration remains at a minimum of 5-8% of methionine intake. Because the transsulfuration pathway is the primary method of disposing of the sulfur moiety of methionine (Finkelstein, 2000, Stipanuk, 1986), the basal rate of transsulfuration is likely maintained simply for methionine catabolism.

In the parenterally fed piglets receiving the highest intake of cysteine (0.5 g kg^{-1} d^{-1}), cysteine synthesis was significantly reduced ($P<0.05$) (Table 3.5.2) compared to the other three intakes above the breakpoint in cysteine synthesis. This further reduction may suggest that there is a secondary mechanism or additional tissue that exhibits inhibition of cysteine synthesis or transsulfuration at the highest dietary cysteine intake. Given that the pancreas and kidney have 90 and 45 percent, respectively, of the specific activity of hepatic CBS (Finkelstein, 1990); we speculate that there was a further reduction in transsulfuration via inhibition of pancreatic or renal CBS which was evident at the highest intake of cysteine (Table 3.5.2). Examination of several higher dietary intakes of cysteine may help to further elucidate the effects of high dietary cysteine intakes on obligatory methionine oxidation in parenterally fed neonatal piglets.

Parenterally fed piglets had a higher obligatory rate of methionine oxidation (8.07 ± 2.02 (SD) % of dose oxidized) than enterally fed piglets (5.16 ± 1.58 (SD) % of dose oxidized). Generally, obligatory amino acid oxidation is greater when first pass splanchnic metabolism is intact (Reeds et al, 2000, Lobley et al, 2003) and is believed to be linked to the energy and synthetic needs of the gastrointestinal tract (Reeds et al., 2000). However, in the case of methionine, parenterally fed piglets had higher obligatory

methionine oxidation than enterally fed piglets. Therefore, during parenteral feeding there may be an additional requirement for cysteine, such as for glutathione, taurine or sulfate synthesis and this may be partially supplied by dietary methionine, resulting in higher levels of methionine oxidation in parenterally fed piglets. Indeed, Mosharov et al. (2000) demonstrated that when there was an increase in oxidants, as commonly observed in parenterally fed neonates (Shahal et al, 1991), the flux of methionine through the transsulfuration pathway increased to enable upregulation of glutathione synthesis in cells challenged by oxidative stress. The higher rate of obligatory methionine oxidation in parenterally fed piglets compared to enterally fed piglets and the amount of cysteine required to reduce methionine oxidation to obligatory levels suggest that our previous estimate of the parenteral total sulfur amino acid requirement (Shoveller et al., 2003a and 2003b; Chapters 3.1 and 3.2) may have underestimated the metabolic requirement for cysteine. If the higher rate of obligatory methionine oxidation in parenterally vs. enterally fed piglets represents differences in the rate of transsulfuration and are due to a demand for increased cysteine synthesis, this additional cysteine does not appear to be required to optimize protein synthesis (Shoveller et al, 2003b; Chapter 3.2), but rather to optimize synthesis of some metabolites, such as glutathione and/or taurine. These metabolic requirements would not have been detected using the indicator amino acid oxidation technique if cysteine use for protein synthesis was preferential to the synthesis of sulfur products of cysteine.

By comparing the differences in obligatory methionine oxidation between routes of feeding, the current study also demonstrated that the gut does not contribute to cysteine synthesis from methionine otherwise the rate of transsulfuration (methionine

oxidation) would have been greater during enteral feeding as compared to parenteral feeding. However, this must be reconciled with the evidence that there is substantial utilization of methionine by the gut. We have previously estimated, by comparing the methionine requirement of enterally and parenterally fed piglets, that methionine utilization by the gut was approximately 30% of methionine intake, whether cysteine was supplied or not (Shoveller et al., 2003a and 2003b; Chapters 3.1 and 3.2). Stoll et al. (1998) demonstrated that only 50% of dietary methionine was detected in the portal blood of piglets and no cysteine was detected. Lobley et al. (2003) demonstrated in lambs that the gastrointestinal tract contributed 10% to whole body methionine oxidation, using plasma kinetic analysis and infusing L-[1-^{13}C]-methionine, and also reported a 11% loss in terms of net absorption across the portal drained viscera. Part of the explanation may be derived from the data showing that enterally fed piglets that were fed methionine, but no cysteine, had significantly higher concentrations of plasma total homocysteine when compared to intravenously fed piglets (Shoveller et al, 2004; Chapter 3.3). An additional part of the explanation may be that methionine is an intermediate in methyl group transfers and the gastrointestinal tract has extensive cellular division and requires large amounts of methyl groups for proliferation (Attaix and Meslin, 1991). Therefore, we suggest that the utilization (loss) of methionine across the intestine without subsequent detection of cysteine in the portal vein is mainly due to the synthesis and export of homocysteine.

In the enterally fed piglets, there was a significant second order polynomial response (P=0.0006; Figure 3.5.4), where cysteine oxidation was initially high, was reduced to a value slightly greater than the total sulfur amino acid requirement (~ 0.25 g

kg^{-1} d^{-1}) and then increased again with higher cysteine intakes. The higher rate of cysteine oxidation at low cysteine intakes was possibly due to the upregulation of CDO. Bagley and Stipanuk (1995) demonstrated that rats fed a diet containing adequate methionine, but no cysteine, had a higher hepatic CDO activity than rats fed a diet containing supplemental cysteine. Bagley and Stipanuk (1994) also demonstrated that rats fed excess protein as casein, had higher CDO activities than rats fed a control diet. Thus, it appears that when cysteine and the total sulfur amino acids limit protein synthesis, cysteine oxidation is increased by up-regulation of hepatic CDO activity. However, as cysteine intake increased and excess amino acids decreased, cysteine oxidation decreased until the requirement for the total sulfur amino acids was met. The increase in cysteine oxidation after the total sulfur amino acid requirement had been met is well supported. Supplemental dietary cysteine, when compared to a basal diet, results in higher activity of hepatic CDO in rats (Daniels and Stipanuk, 1982, Bagley and Stipanuk, 1995). Furthermore, CDO activity increased in a dose-response manner in hepatocytes from rats cultured in either methionine or cysteine supplemented media (Ohta et al., 2000, Kwon et al., 2001). However, it should be noted that when cysteine oxidation was compared among the diets by an ANOVA in the present study, we found no differences (Table 3.5.2). Stipanuk and Rotter (1984) showed that rats fed diets with adequate or excess cysteine did not affect the appearance of $^{13}CO_2$ from C1 and C3 labeled cysteine. The present second order polynomial response detected within this study may only have been detectable because of the high numbers of piglets and dietary cysteine intakes studied. Therefore, both excess amino acids and cysteine can increase cysteine oxidation and this increase in oxidation rate is likely mediated through hepatic

CDO activity and the subsequent oxidation or decarboxylation of cysteine sulfinate. The effect of excess dietary protein and cysteine on sulfur amino acid metabolism needs to be further examined.

Unlike the enterally fed situation, cysteine oxidation in parenterally fed piglets was not affected by cysteine intake ($P>0.05$). Of note is that as cysteine intake increased, it appeared that the variability in the rate of cysteine oxidation between pigs increased (Figure 3.5.6). Parenteral administration of diet and isotope bypasses the regulation of sulfur amino acid metabolism that occurs via both small intestinal and first pass hepatic metabolism. Enzymes and metabolites of cysteine metabolism in non-hepatic tissues of rats show little or no response to changes in dietary protein or sulfur amino acid concentrations (Stipanuk et al., 2002); therefore, parenteral feeding would not be expected to show much of an effect of increasing cysteine intake on cysteine oxidation. The apparent increasing amount of variation associated with increasing dietary cysteine during parenteral feeding should be investigated. Future research could examine how liver first pass metabolism vs. direct delivery of cysteine to the peripheral tissues affects cysteine metabolism.

In the present study, although both enteral and parenteral routes of feeding differed in the effect of dietary cysteine intake on cysteine oxidation, obligatory cysteine oxidation was not different between routes of feeding when compared using a paired t-test. Furthermore, the mean % of cysteine dose oxidized was 17.2 and 17.5 for enteral and parenteral routes of feeding, respectively. The C1 carbon of cysteine is released as CO_2 when cysteine is oxidized to pyruvate and sulfinate or decarboxylated and oxidized to form taurine. The high rate of obligatory cysteine oxidation may be due to hepatic

synthesis of taurine from cysteine. The decarboxylation of cysteine to hypotaurine is catalyzed by pyridoxal 5'-phosphate dependent enzyme, cysteine sulfinate decarboxylase (EC 4.1.1.29) and then further oxidized to taurine (Jacobsen et al., 1964). The present rates of whole body oxidation do not agree with in vitro estimates of hepatic cysteine oxidation from rats fed diets with adequate and excess dietary cysteine which were 41% of dose (Stipanuk and Rotter, 1984); however, there were species and diet differences between these experiments. To our knowledge there have not been *in vitro* or *in vivo* studies similar to ours in any other species than the rat. The splanchnic release of cysteine was consistent with its dietary concentration in an arterial-venous study in rats fed a semi-purified diet containing adequate methionine and cysteine (Garcia and Stipanuk, 1992), suggesting that the gut does not oxidize cysteine to any great extent. Furthermore, Stipanuk et al. (2002) have demonstrated that CDO activity in nonhepatic tissues of rats show little response to changes in sulfur amino acid levels. Therefore, we speculate that the gut releases the majority of dietary cysteine into the portal vein and the liver is the main tissue responsible for cysteine catabolism resulting in no differences in cysteine oxidation between parenteral and enteral routes of feeding.

3.5.5 **Summary**

The present study indicates that in enterally fed piglets, increasing dietary cysteine reduced transsulfuration, as measured by methionine oxidation, until the requirement for the total sulfur amino acids was met and then there was little further change in methionine oxidation. Increasing cysteine intake in enterally fed piglets was associated with cysteine oxidation in a second order polynomial relationship. In parenterally fed piglets, increasing dietary cysteine intake reduced transsulfuration in a

similar fashion to enterally fed piglets, but at the highest intake of cysteine there appeared to be a secondary level of regulation resulting in further down regulation of the rate of transsulfuration. The obligatory methionine oxidation was higher in parenterally as compared to enterally fed piglets, suggesting that in the parenterally fed piglets there may be an additional demand for cysteine synthesis. Finally, cysteine oxidation in parenterally fed piglets was not affected by increases in cysteine intake and appears to be regulated differently in parenterally compared to enterally fed piglets.

3.5.6 **Literature cited**

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4.0 SUMMARY AND GENERAL DISCUSSION

The main objective of this thesis was to examine the impact of the small intestine on sulfur amino acid requirements and metabolism. We first demonstrated that the total sulfur amino acid requirement, as defined by the methionine requirement in the absence of dietary cysteine, was 30% lower in parenterally, compared to enterally, fed neonatal piglets (Shoveller et al, 2003a; Chapter 3.1). Next, we measured the methionine requirement in the presence of excess cysteine and demonstrated that cysteine can furnish 40% of the methionine requirement in both parenterally and enterally fed piglets (Shoveller et al, 2003b; Chapter 3.2). All of these requirements were measured using the indicator amino acid oxidation technique. Furthermore, we compared the percentage of phenylalanine dose oxidized at plateau among the four dietary treatments (Shoveller et al, 2003a and 2003b; Chapter 3.1 and 3.2) and found no differences. Therefore, addition of cysteine to both enteral and parenteral diets did not improve protein synthesis and we can conclude that cysteine is not an indispensable amino acid for protein synthesis in neonatal piglets receiving enteral or parenteral total nutrition.

The enteral total sulfur amino acid requirement and the ability of cysteine to replace part of the methionine requirement closely agreed with previous results which established these requirements using growth, feed efficiency and/or nitrogen balance in young pigs (Shelton et al, 1951, Curtin et al, 1952, Becker et al, 1955, Mitchell et al, 1969, Baker et al, 1969, Roth et al, 1989). To our knowledge this research is the first quantitative measure of the total sulfur amino acid requirement and the ability of cysteine to supply part of the methionine requirement in parenterally fed, neonatal piglets. Our lab has also demonstrated that the lysine (House et al, 1998), threonine (Bertolo et al,

1998) and branched chain amino acid (Elango et al, 2002) requirements are lower in parenterally fed piglets than enterally fed piglets. We have recently shown that the tryptophan requirement is not different between routes of feeding (Cvitkovic et al, 2004); therefore, the differences in parenteral and enteral requirements cannot be accounted for solely by the reduction in protein synthesis due to the gut atrophy associated with parenteral feeding. In total, the small intestine is responsible for a significant proportion of the whole body utilization of many essential amino acids and this may affect whole body availability. Conversely, if intermediates are released, the gut likely plays an important role in the interorgan transport of nutrients.

Errors are frequently made when assessing the maximal proportion of the total sulfur amino requirement that can be provided by cysteine. Understanding that the molecular weight of cysteine is 80 % of that of methionine, the total sulfur amino acid requirement is lower when a correct proportion of the sulfur amino acids are provided. In Chapter 3, we calculated that the mean total sulfur amino acid requirement was 0.42 g kg^{-1} d^{-1} and in Chapter 4 we demonstrated that 40% of that can be furnished by cysteine. This results in methionine supplying 0.25 g kg^{-1} d^{-1} and cysteine replacing 0.17 g kg^{-1} d^{-1} of methionine or supplying 0.14 g kg^{-1} d^{-1} of cysteine. Therefore, the total sulfur amino acids would total 0.39 g kg^{-1} d^{-1} of the total sulfur amino acids and is dramatically lower (70%) than the recommended enteral total sulfur amino acid requirement (NRC, 1998). The NRC (1998) recommendations are extrapolated from data from experiments conducted on older pigs and have used a less bioavailable diet containing fibre and other anti-nutritional factors. Each requirement is represented as a true ileal digestibility; and should be comparable to our estimate of the total sulfur amino acid requirement.

Furthermore, Harte (MSc, Thesis, 2004) compared a variety of methionine: cystine ratios below and above the total sulfur amino acid requirement and found that early weaned pigs fed a semi-synthetic diet supplying the total sulfur amino acids at 75% of the recommended requirement (50 methionine: 25 cystine) had equal growth weights and nitrogen retention as pigs receiving a diet which supplied 100% of the total sulfur amino acid (50 methionine: 50 cysteine). Given that the NRC (1998) clearly states that: "the nutrient requirements in this publication are minimum standards without any safety allowances" and that "professional nutritionists may choose to increase the levels of some more critical nutrients to include "margins of safety" in some circumstances", it appears that the recommendation for the mean total sulfur amino acid requirement may be overestimated. However, we should point out that Sasse and Baker (1974) found that chicks receiving a combination of methionine and cystine grew better than birds fed methionine only; therefore, there may be a need for the exogenous supply of cystine. We measured the total sulfur amino acid requirement with methionine alone; therefore, we may have underestimated the total sulfur amino acid requirement if neonatal piglets require an exogenous source of cysteine for functions other than protein synthesis. Furthermore, addition of dietary sulfate as K_2SO_4 further improved growth of chicks fed ratios of methionine and cystine where methionine was not limiting (Sasse and Baker, 1974). Since most of the minerals that we add to the elemental diets are sulfated, the additional sulfate may have spared part of the cysteine requirement. This further suggests that our predicted requirement may be lower than the recommended total sulfur amino acid intake because the sulfate likely spares part of the cystine requirement and therefore part of the total sulfur amino acid requirement. Nevertheless, given that our

estimate is 30% lower than the NRC (1998) recommendation, and sulfate only improved growth by less than 5% (Sass and Baker, 1974), it is likely that the NRC (1998) recommendation for the mean true ileal digestible requirement for the total sulfur amino acids is overestimated.

We also measured the effect of the above diets, namely enterally fed without (IG) and with (IG+C) cysteine and parenterally fed without (IV) and with (IV+C) cysteine, on plasma cysteine and homocysteine concentrations (Shoveller et al, 2004; Chapter 3.3). Plasma cysteine concentrations were highest in the IV+C group, lower in the IG+C group, lower in the IG group and lowest in the IV group. In addition, increases in dietary methionine intake resulted in a linear increase in plasma cysteine concentrations in enterally but not parenterally fed piglets. Plasma homocysteine concentrations were linearly associated with increases in dietary methionine intake in all groups, but the IG group had significantly higher plasma homocysteine concentrations than all other groups.

In the experiment presented in Chapter 3.3, we only investigated the impact of high dietary intakes of cysteine on plasma homocysteine; therefore, it would be interesting, and more relevant to current neonatal nutrition, to investigate whether lower intakes of cysteine have the same effect on lowering plasma homocysteine in enterally fed piglets. The concentrations of L-cysteine in IV+C and IG+C diets were formulated to provide an L-cysteine intake equivalent to the recommended total sulfur amino acid intake (NRC, 1998). These concentrations far exceed those which are supplied in current infant formulas and neonatal TPN. Hyperhomocysteinemia is associated with an increased risk of ischemic and hemorrhagic stroke in newborn infants and children (Hogeveen et al, 2002, Van Beynum et al, 1999); therefore, lower and more clinically

relevant intakes of cysteine should be investigated as a method of decreasing plasma homocysteine concentrations in newborn infants and children. DiBuono et al (2003) found when a proportion of dietary methionine is replaced with cysteine that transmethylation decreased and remethylation increased at dietary relevant intakes. These effects result in the retention of methionine for protein synthesis. Therefore, we speculate that inclusion of dietary cysteine at dietary relevant intakes would result in lower plasma homocysteine concentrations and equal growth as compared to feeding methionine alone to meet the total sulfur amino acid requirement in neonatal piglets.

In our last study, Chapter 3.5, we fed piglets either enterally or parenterally and looked at the effects of a constant methionine intake and increasing cysteine intakes on both methionine and cysteine oxidation. These data demonstrated that when dietary methionine is held constant at 50% of the total sulfur amino acid requirement, increasing dietary cysteine intake resulted in a reduction in cysteine synthesis or the rate of transsulfuration. Furthermore, when an adequate amount of both methionine and cysteine were provided in the diet, the rate of transsulfuration was higher in parenterally fed piglets than in enterally fed piglets receiving a similar diet. This demonstrates that the gut is <u>not</u> responsible for a large proportion of cysteine synthesis. There was a second order polynomial response in cysteine oxidation to increasing cysteine intakes in enterally fed piglets, but no response in parenterally fed piglets. Furthermore, cysteine oxidation did not differ between routes of feeding. These data clearly indicate that dietary cysteine reduced the rate of transsulfuration.

Stoll et al (1998) demonstrated that 52% of dietary methionine disappeared during first pass intestinal metabolism and that there was no appearance of cysteine in the

portal blood. These results, in combination with our results from Chapters 3.1 and 3.2 (Shoveller et al, 2003a and 2003b, respectively), clearly demonstrate that the gut is responsible for the utilization of 30-50% of the sulfur amino acids, but to what end? Given that Stoll et al (1998) could not detect cysteine in the portal blood, one may speculate that it is cysteine that is being utilized by the enterocyte for synthesis of other products such as glutathione, taurine or sulfate. In addition, increases in methionine intake were directly related to increases in plasma cysteine concentrations in enterally, but not parenterally fed neonatal piglets (Shoveller et al, 2003b; Chapter 3.2); this also suggests that a healthy gut plays a critical role in the synthesis of cysteine from dietary methionine, but not what that role is. The concentration and rate of turnover of glutathione is high in piglets (Jahoor et al, 1995) and Stoll et al (1998) speculated that enteral cysteine is channeled into mucosal glutathione synthesis and thus accounts for the lack of appearance of cysteine in the portal blood. If the gut were responsible for the complete synthesis of cysteine from methionine via the transsulfuration pathway for subsequent incorporation into glutathione, the rate of transsulfuration (methionine oxidation) would be greater during enteral than parenteral feeding. In Chapter 3.5, we demonstrated that enterally fed piglets have a lower rate of transsulfuration than parenterally fed and piglets and therefore, the gut is not responsible for complete cysteine synthesis.

Alternatively, the gut may metabolize methionine to another product (i.e. homocysteine) and release it into the portal circulation. Stoll et al (1998) did not measure homocysteine. Furthermore, these researchers' analyzed amino acids in whole blood using phenylisothiocyanate derivatives (PicoTag, Waters, Woburn, MA) which is

not a method designed to measure total cyst(e)ine concentrations. Therefore, it is possible that the gut exported homocysteine and this would have resulted in a net utilization of methionine. It is well established that all cells in the mammalian body have an intact transmethylation/remethylation pathway. The gastrointestinal tract has extensive cellular division and would likely require large amounts of methyl groups for proliferation (Attaix and Meslin, 1991). Therefore, we speculate that the gut exports increased amounts of homocysteine when methionine alone is provided than when a combination of methionine and cysteine are provided. Furthermore, it is possible that the linear association of methionine intake to plasma cysteine was a result of the transsulfuration of homocysteine and subsequent synthesis of cysteine by the liver. To evaluate this theory one can examine the differences between a diet supplying methionine only and a diet supplying a combination of methionine and cysteine, using labeled L-[1-^{14}C]methionine and the portal balance approach. One would expect higher levels of labeled homocysteine and lower levels of labeled methionine in the piglets receiving the diet providing methionine only than in piglets receiving the diet providing a combination of methionine and cysteine. Why this homocysteine is not either remethylated or transsulfurated by the liver or removed by the kidney (as evidenced by the significantly greater homocysteine concentrations seen in IG fed pigs with no dietary cysteine) we cannot speculate on and requires further examination.

This final experiment (Chapter 3.5) did not include diets that provided methionine at 100% of total sulfur amino acid requirement and methionine in excess of the total sulfur amino acid requirement. This experiment would measure the "normal" rate of transsulfuration (100% methionine, no cysteine) and the rate of transsulfuration in

response to excesses of the sulfur amino acids (cysteine in excess of the total sulfur amino acid requirement with graded levels of dietary methionine starting at the methionine requirement). Comparison of "normal" transsulfuration rates between routes of feeding may indicate why plasma cysteine was significantly lower during parenteral as compared to enteral feeding. If methionine oxidation was higher or equal during parenteral compared to enteral feeding at this level, one could conclude that it is not a limitation in the transsulfuration pathway that results in low plasma cysteine concentrations during parenteral feeding. Second, feeding excess cysteine and feeding graded levels of methionine above the total sulfur amino acid requirement may help elucidate the mechanism of methionine toxicity. One would expect that high intakes of methionine, when dietary cysteine is provided in excess, would increase the rate of transsulfuration to dispose of the excess methionine. Addition of other tracers, such as the doubly labeled methionine would further advance our knowledge of the *in vivo* regulation of the methionine cycle and transsulfuration under these feeding conditions in the neonate. Specifically, doubly labeled methionine would allow measurement of the rate of transmethylation and remethylation in addition to transsulfuration, which we have already measured. High dietary intakes of methionine, with no dietary cysteine, will likely cause an increase in transmethylation and a decrease in remethylation which would result in an increase in homocysteine and upregulation of the transsulfuration pathway. Conversely, addition of cysteine (or replacement of methionine) will likely decrease transmethylation and increase remethylation, resulting in retention of methionine and decreased plasma homocysteine concentrations as compared to diets supplying methionine only. However, when cysteine is provided and methionine is in excess, the

effects of cysteine on transmethylation and remethylation may be eliminated in order to dispose of methionine via the transsulfuration pathway. The regulation of transmethylation, remethylation and transsulfuration by the dietary supply of methionine and cysteine still requires work and can also be applied to examining the kinetics of the sulfur amino acids during parenteral feeding and disease states, such as cholestasis, where the sulfur amino acids have been implicated in playing a critical role (Moss et al, 1999).

Intravenous methionine infusion is related to decreased bile flow and it is believed that this may precede cholestasis (Moss et al, 1999). More recent evidence suggests that infusion of homocysteine increases bile flow without any liver damage (Belli et al, 2003). Interestingly, providing methionine as the sole amino acid to piglets receiving parenteral feeding produces significantly lower plasma homocysteine concentrations than enteral feeding (Shoveller et al, 2004; Chapter 3.3). This reduction in plasma homocysteine during parenteral feeding may play a prominent role in the onset of cholestasis, rather than dietary methionine alone. It would be interesting to determine how intravenous infusion of homocysteine, as a partial replacement for methionine, would affect hepatic cholestasis, and sulfur amino acid metabolism in the parenterally fed neonate and whether this would also increase cysteine concentrations. Clearly, the role of the low plasma homocysteine and cysteine concentrations during parenteral feeding on parenteral nutrition induced cholestasis is an interesting topic that requires further investigation.

Although we defined the sulfur amino acid requirements, characterized the effects of parenteral and enteral sulfur amino acid intake on cysteine and homocysteine, and

demonstrated that parenterally fed piglets have a higher rate of cysteine synthesis but equal cysteine oxidation, we have been unable to answer why plasma cysteine concentrations remain low in parenterally fed piglets that are not receiving high intakes of cysteine. A possible answer is that parenterally fed neonates utilize more cysteine for glutathione synthesis. Harte (MSc. Thesis, 2004) quantified the tissue glutathione concentrations and the incorporation of 1-[^{14}C] cysteine into glutathione in jejunal, hepatic and renal tissue of enterally fed piglets receiving 0.25 g methionine kg^{-1} d^{-1} and graded intakes of cysteine and found that increasing cysteine results in an increase in hepatic glutathione concentrations, no changes in intestinal or renal glutathione concentrations and no effect on the incorporation of cysteine into glutathione in any tissue. Measurement of these same parameters in parenterally fed piglets and subsequent comparison to the enterally fed piglets from Harte (MSc thesis, 2004) will elucidate whether glutathione concentrations or synthesis are higher in neonatal piglets receiving parenteral nutrition.

S-nitrosation has been suggested as a possible storage mechanism for nitric oxide (Alencar et al, 2003) and involves the transfer of nitric oxide from one cysteine residue to another. These researchers exposed rat and porcine coronary artery *in vitro* to nitrosoglutathione which resulted in an increase in cysteine-nitric oxide residues and an increase in nitric oxide. Furthermore, addition of N-acetyl-L-cysteine displaces nitric oxide from the cysteine residues (Alencar et al, 2003). These researchers concluded that S-nitrosated tissue thiols may serve as a local storage from which bioactive nitric oxide can be released. Interestingly, arginine supplementation has been examined as a therapy during disease states, such as necrotizing entercolitis (Di Lorenzo et al, 1995). Cynober

(1994) has suggested that arginine would act as a nitric oxide precursor and that increased nitric oxide would enhance immune function. However, Castillo et al (1995) estimated that only approximately 1% of arginine flux is utilized for nitric oxide synthesis and even if this doubled during disease states or stress, it is unlikely that this would account for a significant proportion of cysteine. However, if cysteine binds to nitric oxide in an attempt to mediate the stress associated with parenteral feeding and thus increase nitric oxide synthesis and availability, it could present a possible outlet for cysteine or it could mean that we (and others) could not detect this pool of cysteine.

In Chapter 3.4 we demonstrated that N-acetyl-L-cysteine supported equal growth and protein deposition as the control diet, which supplied L-cysteine. Cysteine is unstable in solution, parenteral feeding results in a significant reduction in plasma cysteine and N-acetyl-L-cysteine supports equal growth and protein deposition as equimolar amounts of L-cysteine; therefore, N-acetyl-L-cysteine should be considered for inclusion in neonatal total parenteral nutrition as a stable, Maillard resistant precursor for cysteine. Prior to being used clinically, the effectiveness of N-acetyl-L-cysteine for human neonates needs to be evaluated.

The effects of N-acetyl-L-cysteine (highNAC) compared to isomolar amounts of L-cysteine on tissue free amino acid concentrations produced some interesting differences. In liver, jejunum and kidney, methionine concentrations were consistently lower in the control piglets than the highNAC piglets. However, the muscle methionine concentrations were greater in the control pigs. Second, taurine concentrations were higher in the control diet than the highNAC diet in all tissues. N-acetyl-L-cysteine may act as an antioxidant and this is likely the effect of the greater partitioning of N-acetyl-L-

cysteine to glutathione rather than taurine as compared to diets which supplied cysteine (Banks and Stipanuk, 1994). The dietary provision of cysteine or N-acetyl-L-cysteine may produce similar rates of growth and nitrogen retention; however, N-acetyl-L-cysteine and L-cysteine are clearly metabolized differently and the impact of these differences should be investigated in neonates receiving parenteral nutrition. Future studies should examine the effects of N-acetyl-L-cysteine on glutathione concentrations and synthesis and tissue methionine concentrations and metabolism. To accomplish this, tissue and plasma concentrations of glutathione and taurine can be compared between piglets receiving parenteral diets containing L-cysteine or N-acetyl-L-cysteine. We speculate that piglets receiving N-acetyl-L-cysteine will have higher glutathione concentrations and decreased taurine concentrations as compared to diets supplying L-cysteine.

What was also clear from the combination of Chapter 3.1, 3.2 and 3.3 is that plasma methionine concentrations do not seem to be affected by increasing sulfur amino acid intake. We speculated that methionine concentrations likely changed intracelluarly; however, we did not measure tissue concentrations in every experiment. Potgieter et al (1997) demonstrated that plasma methionine concentrations need to be corrected for oxidative losses during storage and processing to account for the oxidative product of methionine, methionine sulfoxide. Our sample preparation methods are not substantially different; however, these researchers used a different reverse phase chromatography (*o*-phthalic aldehyde mercaptoethanol derivatization) and fluorescence detection. In the future, it is important that studies using plasma methionine as a dependent variable be

corrected for these oxidative losses if plasma methionine is going to be used as a dependent variable.

Although we detected no differences in plasma methionine among dietary intakes of methionine in parenterally and enterally fed piglets, plasma taurine concentrations changed, which suggests that taurine synthesis is not limited in the neonatal piglet as suggested by some researchers (for review: Lorenco and Camilo, 2002). Either the abundance or activity of cysteine sulfinate decarboxylase is different between human neonates and piglets, or there is not a limitation in taurine synthesis in the neonate. To address this, cysteine sulfinate abundance and activity should be measured in neonatal piglet tissue. In addition, the plasma amino acid concentrations in the final study will be measured to ascertain whether increasing the total sulfur amino acid intake, via increasing cysteine intake (as in Chapter 3.5) and not methionine intake (as in Chapter 3.1, 3.2 and 3.3) have the same effect on taurine production. Furthermore, the partitioning of cysteine between taurine production (C1 carbon release) and pyruvate production (C1 and C3 carbon release) may be affected by changes in the sulfur amino acid ratio.

It was evident in Chapter 3.2 (Shoveller et al, 2003b) that taurine production seemed to be mediated by both excess amino acid catabolism (below the breakpoint for methionine) and by cysteine/methionine intake above the breakpoint. These plasma taurine data are congruent with the cysteine oxidation data in enterally fed piglets (Chapter 3.5) but the regulation of cysteine oxidation and production of taurine or pyruvate is unclear in the parenterally fed situation and requires further examination. Specifically, the plasma taurine concentrations were greater when cysteine was in excess

but methionine was limiting; as methionine intake increased, taurine concentrations decreased until protein synthesis was maximized; after protein synthesis was maximized and methionine intake was further increased, taurine concentrations increased again in both parenterally and enterally fed piglets. Although the cysteine oxidation and plasma taurine concentrations are congruent in enterally fed piglets, they are not in parenterally fed piglets. The partitioning of cysteine between taurine and pyruvate production should be examined during parenteral feeding.

Although we can conclude that dietary cysteine reduces the methionine requirement, the fate of the ~ 5% obligatory methionine oxidation remains unknown. Two possibilities exist. First, this is the basal amount of methionine that must be shuttled through the transsulfuration pathway to produce cysteine, possibly as a constant outlet for the highly toxic methionine and because enzyme activity cannot completely cease. It would be interesting to determine if at higher intakes of methionine (1-3 times the requirement), the transsulfuration pathway was further upregulated to enable additional disposal of methionine. Feeding graded levels of methionine above the total sulfur amino acid requirement and measuring methionine oxidation could answer this question. Second, this obligatory methionine oxidation may be due to the decarboxylation of S-adenosylmethionine to produce decarboxylated adenosylmethionine for donation of an aminopropyl group to polyamine synthesis. Giulidori et al (1984) measured *in vivo* rates of methionine oxidation in rats and suggested that 30% of methionine oxidation was used for aminopropyl donation and 70% of methionine oxidation was catabolized via the transsulfuration pathway. Indeed, at the lowest intake of dietary cysteine we found that methionine oxidation was approximately 25% of dose oxidized and at adequate total

sulfur amino acid intakes (above the breakpoint), methionine oxidation was approximately 5% of dose oxidized. This suggests that obligatory methionine oxidation is approximately 20% of total *in vivo* methionine oxidation and compares to Giulidori et al (1984) suggesting that the polyamine pathway may account for the obligatory methionine oxidation; however, further research is required to determine the partitioning of S-adenosylmethionine between polyamine synthesis and transmethylation in the neonatal piglet. All of these possibilities could be complemented by examining the enzyme activities of these different and distinct pathways.

In preliminary studies to examine the bioavailability of N-acetyl-cysteine, we set the methionine intake at 0.18 g $kg^{-1}d^{-1}$, the mean parenteral methionine requirement determined when cysteine was in excess, and administered intakes of N-acetyl-L-cysteine (same N-acetyl-L-cysteine intake as zeroNAC, lowNAC and highNAC in Chapter 6) or L-cysteine as the control. In the following study, Chapter 3.4, piglets were administered methionine at the 95% confidence interval intake, 0.30 g $kg^{-1}d^{-1}$ (Shoveller et al, 2003b: Chapter 3.2). All of the piglets in the preliminary experiment (n=2 per diet level) became hyperammonemic 5 days after initiation of test diet. After receiving a bolus dose of methionine, 5 out of 8 pigs recovered and 3 had to be euthanized. In addition, at necropsy there were signs of pars esophageal ulcers in all 8 piglets and 3 piglets had bleeding pars esophageal ulcers. The most fascinating aspect of this event was that even though all the pigs were receiving different intakes of N-acetyl-L-cysteine or cysteine and equal intakes of methionine, all became hyperammonemic within hours of each other and 5 days after test diet initiation. This evidence suggests that the intake of methionine was the cause of both the hyperammonemia and the gastric ulcers,

independent of the cysteine source or level. In addition to the hyperammonemia and gastric ulcers that were observed, 5 out of 8 of these piglets retained significant amounts of fluid within the first couple days of test diet initiation. Interestingly, Sasse and Baker (1974) found that inclusion of sulfate in chick diets with a 60:40 methionine to cysteine ratio, resulted in greater weight gains and reduced body water than chicks receiving no sulfate. It is possible that this methionine deficiency produced a step wise progression in deficiency symptoms. The water retention occurred first and may have been due to limited sulfate synthesis; a reduction in growth rate was noted second with some pigs having trouble with movement; lastly, the piglets became hyperammonemic. Therefore, there was likely a deficiency in sulfate, methionine and methyl groups. The deficiency in methionine with concurrent administrations of all other dispensable and indispensable amino acids would have eventually overwhelmed the urea cycle and would have ultimately resulted in hyperammonemia. The most surprising of these results is the occurrence of gastric ulcers. It would be interesting to look at enteral diets with limiting (low) concentrations of methionine (as all these results occurred in parenterally fed animals) and see if similar gastric lesions are found. Furthermore, although higher levels of methionine are related to cholestasis, this evidence suggests that low levels are related with hyperammonemia and gastric ulcer formation. However, it is unclear which event was primary and the gastric ulcers may simply be an indication of metabolic stress. The occurrence of hyperammonemia is also surprising, considering that the piglets were all getting different intakes of N-acetyl-cysteine or L-cysteine and therefore different intakes of the total sulfur amino acids. This indicates that there was no effect of cysteine sparing on the methionine requirement for protein synthesis or that the hyperammonemia was not

caused by a deficiency of the sulfur amino acids for protein synthesis and excess catabolism of the other amino acids. Although this thesis has provided some important information as to methionine and cysteine ratios in diets, further research is still necessary to determine not only the requirements for protein synthesis but the sulfur amino acid requirements for non-protein synthetic activities. It may be that the ratio is different for protein synthesis than it is for the non-protein synthetic functions of these two amino acids.

This discussion would be incomplete without mention that sulfur amino acid metabolism is affected by many other metabolites, such as vitamin cofactors (vitamin B-12, folate, betaine, and vitamin B-6), amino acids (serine and glycine), intermediates of the methionine cycle and transsulfuration, and other aspects that may affect sulfur amino acid metabolism, such as methyl demand. Because we provided equal intakes of these vitamins, and serine and glycine, we assume that these did not affect any of our results. Investigation of the relationship among these other metabolites and the sulfur amino acid in porcine and human neonates should be conducted to determine if it is different than in growing and adults rats.

The series of experiments presented in this thesis have demonstrated that the neonatal small intestine is critical to sulfur amino acid metabolism and whole body homeostasis. Clearly, by-pass of the small intestine appears to perturb cysteine metabolism and although we have advanced the understanding of role of the intestine, further research will elucidate the mechanism for this perturbation. Such findings have significant implications in neonatal populations in which small intestinal first pass

metabolism is by-passed or compromised. There are a great many possibilities for future research in this area.

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